University of Alberta

The Development and Characterization of CD1a+ Dendritic Cells from CD34+ Progenitor Cells Isolated from Human Umbilical Cord Blood

by

Julie O. Theng

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Experimental Surgery

Department of Surgery

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **The Development and Characterization of CD1a+ Dendritic Cells from CD34+ Progenitor Cells Isolated from Human Umbilical Cord Blood** submitted by **Julie O. Theng** in partial fulfillment of the requirements for the degree of **Masters of Science** in **Experimental Surgery.**

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ABSTRACT

Background-Dendritic cells are the most potent antigen presenting cells and may be useful in cancer immunotherapy. We wished to generate DCs from CD34+ progenitors and examine the effects of CpG adjuvant.

Methods-CD34+ progenitor cells from cord blood were cultured in GM-CSF, TNF-α and SCF and assessed morphologically. Flow cytometry determined surface phenotype and endocytic activity. The DCs were used to study the effect of CpG adjuvant on T-cell proliferation.

Results-Initial levels of 84.5% ± 4.5 CD34 on progenitor cells disappeared by day 10. CD1a expression appeared at day 7 and reached 89.2% ± 1.2 by day 20. CD83 expression was evident by day 14 and reached 28.7% ± 6.5 by day 24. On day 10, 33.6% of cells engulfed particulate antigen. CpG adjuvant increased T-cell proliferation, perhaps non-specifically

Conclusions-CD1a+ DCs can be reproducibly generated from CD34+ progenitor cells in cord blood. CpG adjuvant causes a marginal increase in T-cell stimulation.

For Mom and Dad, thank you for everything

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TABLE OF CONTENTS

CHAPTER ONE: GENERAL INTRODUCTION 1

Cancer Immunotherapy 1

Dendritic Cells 4

Lymphoid versus Myeloid Dendritic Cells 5

Characterization of Dendritic Cells 7

In Vivo Action of Dendritic Cells 9

DC Recruitment 9

Antigen Uptake 9

Dendritic Cell Migration 10

Antigen Processing and Presentation by Dendritic Cells 11

T-cell Activation by Dendritic Cells 12

Dendritic Cell Based Cancer Immunotherapy 13

CpG Motif Oligodeoxynucleotide 16

History 16

Comparisons between Vertebrate and Bacterial DNA 18

Development of the CpG Motif Oligodeoxynucleotide 19

Immunostimulatory Effects of CpG Motif Oligodeoxynucleotide 21

B-cells 21

NK Cells 21

T-cells 22

APCs 23

Th1 Response 24

Mechanism of Action of CpG Motif Oligodeoxynucleotide 25

Purpose of this Investigation 26

CHAPTER TWO: MATERIALS AND GENERAL METHODS 28

Materials 28

General Methods 30

Isolation of CD34+ Progenitor Cells from Human Cord Blood 30

Obtaining Mononuclear Cells 30

Immuno-magnetic Labeling of CD34+ Progenitor Cells 30

Release of CD34+ Progenitor Cells 31

Culture of CD34+ Progenitor Cells 31

T-Cell Isolation using Sheep Red Blood Cells 31

Preparation of Neuraminidase Treated Sheep Red Blood Cells 31

Flow Cytometry 32

CHAPTER THREE: ISOLATING CD34+ PROGENITOR CELLS FROM HUMAN UMBILICAL CORD BLOOD 33

Introduction 33

Sources of Dendritic Cells 33

Immuno-magnetic Labeling: Positive and Negative Selection 34

Purpose of this Investigation 35

Materials and Methods 36

Results 36

The Effect of Cord Blood Volume on Mononuclear Cell and CD34+ Progenitor Cell Yields 36

The Effect of Cord Blood Age on Mononuclear Cell and CD34+ Progenitor Cell Yields 37

Discussion 38

CHAPTER FOUR: THE DEVELOPMENT AND CHARACTERIZATION OF DENDRITIC CELLS USING MORPHOLOGY, CELL SURFACE PHENOTYPE AND FUNCTION 51

Introduction 51

Development of Dendritic Cells 51

Characteristics of Dendritic Cells 52

Purpose of this Investigation 55

Materials and Methods 56

Examination of Dendritic Cell Morphology and Cell Surface Phenotype 56

Examination of Antigen Uptake by Dendritic Cells 57

Preparation of Stained Necrotic LT210 Cells 57

Co-culture of CD1a+ Cells and Stained Necrotic LT210 Cellular Fragments 58

Flow Cytometry 58

Results 59

Examination of Dendritic Cell Morphology and Cell Surface Phenotype 59

Examination of Antigen Uptake by Dendritic Cells 61

Discussion 61

CHAPTER FIVE: THE EFFECT OF CpG MOTIF OLIGODEOXYNUCLEOTIDE ON ANTIGEN PRESENTATION BY DENDRITIC CELLS 73

Introduction 73

Dendritic Cell Based Cancer Immunotherapy 73

CpG Motif Oligodeoxynucleotide 75

Purpose of this Investigation 78

Materials and Methods 78

T-cell Proliferation Assay 78

Preparation of CD1a+ Cells (Stimulators) and LT210 Necrotic Fragments 78
Preparation of T-cells (Responders) 79
Set Up of T-cell Proliferation Assay (Stimulators + Responders) 79
Modified T-cell Proliferation Assay: 24 Hour Incubation of Stimulators and LT210 Necrotic Fragments in 96 Well Plates 81
Results 82
T-cell Proliferation Assays 83
Autologous T-cell Proliferation Assay in Fetal Bovine Serum 83
Autologous T-cell Proliferation Assay in Human Serum Type AB 84
Allogenic T-cell Proliferation Assay in Human Serum Type AB 86
Discussion 87

CHAPTER SIX: GENERAL DISCUSSION 101

REFERENCE LIST 111

LIST OF TABLES

TABLE

5-1. The Counts per Minute representing the Degree of T-cell Proliferation in an Autologous T-cell Proliferation Assay using Media supplemented with FBS 93

5-2. The Counts per Minute representing the Degree of T-cell Proliferation in an Autologous T-cell Proliferation Assay using Media supplemented with Human Serum Type AB 94

5-3. The Counts per Minute Representing the Degree of T-cell Proliferation in an Autologous T-cell Proliferation Assay using Media supplemented with Human Serum Type AB in a Volume of 175 µl 95

5-4. The Counts per Minute representing the Degree of T-cell Proliferation in an Allogenic T-cell Proliferation Assay using Media supplemented with Human Serum Type AB 96

LIST OF FIGURES

FIGURE

3-1. Mean Counts of Total Number of Mononuclear Cells Isolated from Human Cord Blood and Analyzed According to Blood Sample Volumes 42

3-2. Mean Counts of Mononuclear Cells/ml Isolated from Human Cord Blood and Analyzed According to Blood Sample Volumes 43

3-3. Mean Counts of Total CD34+ Progenitor Cell Counts Isolated from Human Cord Blood and Analyzed According to Blood Sample Volume 44

3-4. Mean Counts of CD34+ Progenitor Cells/ml Isolated from Human Cord Blood and Analyzed According to Blood Sample Volume 45

3-5. Mean Counts of Total Mononuclear Cells Isolated from Human Cord Blood and Analyzed According to Time Elapsed Between Sample Collection and Sample Processing 46

3-6. Mean Counts of Mononuclear Cells/ml Isolated from Human Cord Blood and Analyzed According to Time Elapsed Between Sample Collection and Sample Processing 47

3-7. Mean Counts of Total CD34+ Progenitor Cells Isolated from Human Cord Blood and Analyzed According to Time Elapsed Between Sample Collection and Sample Processing 48

3-8. Mean Counts of CD34+ Progenitor Cells/ml Isolated from Human Cord Blood and Analyzed According to Time Elapsed Between Sample Collection and Sample Processing 49

3-9. A Flow Cytometry Histogram for a Representative Sample of CD34+ Progenitor Cells 50

4-1. Experimental Design for Assessment of Antigen Uptake by DCs. 67

4-2. CD34+ Progenitor Cells Cultured between 0 and 14 Days in Media Supplemented with GM-CSF and TNF-α 68

4-3. The Mean Cell Numbers Derived from CD34+ Progenitor Cells Isolated from Human Cord Blood and Cultured for 30 Days 69

4-4. Flow Cytometry Histograms for a Representative Sample of CD34+ Progenitor Cells Isolated from Human Cord Blood and Cultured for 30 Days 70

4-5. Mean Percent Expression of CD34, CD1a and CD83 on CD34+ Progenitor Cells Isolated from Human Cord Blood and Cultured for 30 Days in the Presence of GM-CSF and TNF-α 71

4-6. The scatter plots showing fragment engulfment versus the adhesion of fragments on the outside cell surface. 72

5-1. The Stimulation Indices of Autologous T-cell Proliferation Assay Conducted in the Presence of FBS. 97

5-2. The Stimulation Indices of Autologous T-cell Proliferation Assay Conducted in the Presence of Human Serum Type AB 98

5-3. The Stimulation Indices of Autologous T-cell Proliferation Assay Conducted in the Presence of Human Serum Type AB in a Volume of 175 µl 99

5-4. The Stimulation Indices of Allogenic T-cell Proliferation Assay Conducted in the Presence of Human Serum Type AB. 100

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
BCG	Bacillus Calmette-Guérin
CCR	CC Chemokine Receptor
CFA	Complete Freund's Adjuvant
CFDA SE	Carboxyfluorescein Diacetate Succinimidyl Ester
CFSE	Carboxyfluorescein Succinimidyl Ester
CD	Cluster of Differentiation
CpG	Cytosine-Phosphodiester Bond-Guanine
CTL	Cytotoxic T-Lymphocyte
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific, ICAM-3 Grabbing, Nonintegrin
DNA	Deoxyribonucleic Acid
FACS	Fluorescence Activated Cell Sorter
FLT3L	fms-like Tyrosine Kinase 3 Ligand
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
IB	Isolation Buffer
ICAM-I	Intracellular Adhesion Molecule
IFN	Interferon
IgG	Immunoglobulin
IL	Interleukin
KLH	Keyhole Limpet Hemocyanin
LCs	Langerhan Cells
LFA	Leukocyte Function Associated Antigen
MAPK	Mitogen Activated Protein Kinase
MCP	Monocyte Chemoattractant Protein
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II
MIP	Macrophage Inflammatory Protein
MLR	Mixed Lymphocyte Reaction
MNC	Mononuclear Cell
NF-κB	Nuclear Factor Kappa B
NK	Natural Killer
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PHA	Phytohemagglutinin
PSGL	P-Selectin Glycoprotein Ligand
RANK	Receptor Activator of NFkB Ligand
RANTES	Regulated upon Activation, Normal T-cell Expressed and Presumably Secreted

rER	rough Endoplasmic Reticulum
SCF	Stem Cell Factor
SI	Stimulation Index (Test Condition/Background Condition)
TAP	Transporters Associated with Antigen Processing
Th1	T-cell Helper 1
Th2	T-cell Helper 2
TCR	T-cell Receptor
TGF	Tumor Growth Factor
TIL	Tumor Infiltrating Lymphocyte
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
TSA	Tumor Specific Antigen

CHAPTER ONE: GENERAL INTRODUCTION

Cancer Immunotherapy

Cancer remains an emotionally and physically painful disease that can significantly reduce not only the quality of life, but also the life span of an individual. According to statistics released by the Canadian Cancer Society in 2002, cancer is the Nations leading cause of premature death being diagnosed in 38% of women and 41% of men during their lifetimes. The current conventional method of treating cancer involves a multi-modal strategy that includes all, or combinations of, surgery, chemotherapy, and radiation therapy. Surgery is a limited therapy for obvious reasons including invasiveness, inaccessibility of tumor sites and the high likelihood of leaving residual tumor cells after surgical resection. Chemotherapy and radiation therapy can be effective, but many tumors remain unresponsive to these modes of therapy and both these methods have toxic side effects that are undesirable in already ailing patients [2,3]. In consequence, new methods of treating this debilitating disease are constantly under examination, and one such area is cancer immunotherapy.

Cancer immunotherapy is in essence, the stimulation of a host immune response against cancer. The immune system has become an attractive focal point for cancer therapy in response to the effectiveness with which tumors evade the immune system. Some general tactics that tumors are postulated to employ include growing in partially immuno-privileged sites and secreting immunosuppressive factors such as tumor growth factor (TGF)-β and interleukin (IL)-10 [4]. More specific evasion mechanisms involve the down regulation of the major histocompatibility complex (MHC) I pathway and co-stimulatory molecules B7-1 and B7-2 on the surface of the tumor cells. These cell

surface markers are important in T-cell recognition and activation. As well, tumors have been found to decrease the cell surface expression of immunogenic tumor antigens resulting in the absence of an effective immune response [4,5]. T-cells may also have difficulties in responding to tumor cells appropriately. For example, T-cells may undergo apoptosis upon contact with tumor cells due to tumor-induced degradation of the zeta chain [4,6] and there may be an inadequate number of anti-tumor T-cells present [4].

Attempts to circumvent these mechanisms of evasion led to several ideas, one of which revolved around the potential use of cytokine therapy. An example of this was the direct administration of IL-2 into mice bearing tumors. IL-2 is a cytokine that acts as a T-cell growth factor and mediates the expansion of T-cells in vivo [4]. Upon administration of IL-2 to these mice, tumor regression was observed [7]. In humans, a similar effect was observed where IL-2 administration mediated the regression of human cancers with metastatic melanomas, metastatic kidney cancers and B-cell lymphomas being especially susceptible [4]. 15% of patients with metastatic melanoma, and 19% of patients with metastatic kidney cancer experienced tumor regression with roughly half in each of these groups experiencing total remission [4]. The results of this, and other similar studies led to the approval of high-dose IL-2 treatment of both metastatic melanoma and metastatic kidney cancer patients by the American Food and Drug Administration [4].

Other more recent studies have focused on the administration of cytokines through more indirect methods. Parney et al. used retroviral vectors to transduce granulocyte macrophage-colony stimulating factor (GM-CSF) and B7-2 genes into the human glioblastoma cell line D54MG. GM-CSF is a growth factor that has many effects, in particular, it acts on APCs, while B7-2 is a co-stimulatory molecule required for T-cell

activation. Vaccinating the allogenic-human-lymphocyte-human glioblastoma severe combined immunodeficiency (SCID) mouse model with the transduced cells after ensuring adequate levels of expression of GM-CSF and B7-2 resulted in an inhibition of wild type tumor growth at distant sites [8]. Another example involved the use of biolistic transfection methods to deliver the IL-12 gene into epidermal cells present directly over an intradermal tumor. IL-12 is a factor that is involved in the stimulation of natural killer (NK) cells, promotes the maturation of cytotoxic T-lymphocytes (CTLs) and has also been shown to elicit anti-tumor effects in mice [9,10]. An anti-tumor response was exhibited in these experiments [9,10].

A strategy different from those cytokine therapies mentioned above involved the use of tumor infiltrating lymphocytes (TILs). TILs grow within the stroma of tumors, and when isolated from mice were capable of recognizing tumor antigens in *in vitro* assays [11]. TILs isolated from human tumors exhibited an anti-tumor response *in vitro* [12]. These results led to the development of clinical trials where patients with metastatic melanoma were treated with autologous TILs obtained from the tumor site and expanded *in vitro* with IL-2 [13,14]. Approximately 34% of patients treated with these methods responded to the therapy [15].

Presently, an area of cancer immunotherapy that is receiving much attention is the identification of tumor specific antigens (TSA). The idea behind this method is that if the tumor expresses tumor specific antigens that are truly limited to expression on the tumor, the immune system will be able to recognize these antigens and launch an immune response only towards the tumor cells [16]. The key to this strategy is the use of antigen presenting cells (APC), which, when pulsed or fed with TSA, can present these antigens

to T-cells and thereby initiate an immune response. One APC that has received much attention is the dendritic cell (DC), which is the most potent APC identified, and has given rise to a new field of cancer immunotherapy called DC based cancer immunotherapy.

Dendritic Cells

DCs were first visualized in 1868 as Langerhan cells in the skin. However, they were not officially characterized until 1973, when Steinman and Cohn isolated DCs from the lymphoid organs of mice [17,18]. These cells were termed "dendritic" due to the presence of numerous protrusions on the cell surface, which resembled the dendrites of nerve cells [17].

Initial attempts to investigate the properties of DCs were hampered by the inability to isolate DCs in large homogenous quantities, the short life span of these cells in culture and the absence of specific cell surface markers to identify DCs [18,19]. New advances in scientific research have helped investigators circumvent some of these difficulties. DCs are now commonly isolated from peripheral blood or umbilical cord blood (this topic will be discussed in further detail in Chapter 3) and have now been discovered to play a pivotal role in the immune system serving as a bridge between the innate and adaptive immune system [20].

DCs are the most potent antigen presenting cells, the most endocytically active cells and the only APC capable of eliciting a primary immune response. [21]. That is, by presenting antigen via MHC I or MHC II to CD8+ or CD4+ T-cells respectively, they can cause the activation and proliferation of naïve T-cells [21].

Another important feature of DCs is their ability to "cross prime." Traditionally, endogenous antigen was thought to be exclusively presented on MHC I which activated CD8+ T-cells that went on to generate a CTL immune response. Conversely, exogenous antigen taken up by APCs was thought to be exclusively presented on MHC II which went on to activate CD4+ T-cells [21,22,23]. In the case of cross priming, APCs are capable of presenting endocytosed exogenous antigen in the presence of MHC I to activate CD8+ T-cells and elicit a CTL response [22]. This ability is important for the activation of killer T-cells towards viral and tumor antigens [21]. DCs are better than any other APC at cross priming [22].

Lymphoid Versus Myeloid Dendritic Cells

In the mouse, two distinct pathways of DC development have been observed: the lymphoid path, and the myeloid path [20]. Lymphoid and myeloid derived DCs can be distinguished by slight differences present in cell surface phenotype, location and function [16,20,24]. Both types of DCs express CD11c, MHC II, CD86 and CD40 however lymphoid DCs can be identified very reliably by the cell surface marker CD8α which is absent on the surface of myeloid DCs [20]. Lymphoid DCs can be found in T-cell rich areas of the periarteriolar lymphatic sheath (PALS) in the spleen and lymph nodes whereas myeloid DCs are found in the marginal zone, which bridge channels of the spleen [20]. Functionally, lymphoid DCs are more phagocytic than myeloid DCs, however, myeloid DCs make more IL-12, an immuno-stimulatory cytokine, and prime CD4+ and CD8+ T-cells more efficiently than lymphoid DCs [20].

In humans, myeloid and lymphoid DCs are not as clearly defined as in the mouse. In fact, the actual existence of lymphoid derived DCs is controversial [20]. What is thought

to be the lymphoid equivalent in humans are CD11c negative (CD11c-) DCs that have low endocytic capacity [20]. One proposed role of the CD11c- DCs in humans is to induce tolerance, especially since an abundance of these DCs have been found in the thymus [23,25]. These cells are not completely understood however, some reports have shown that although the CD11c- DCs may induce tolerance in the thymus, they have also been shown to prime naïve T-cells in the periphery [16]. Human myeloid DCs on the other hand, have been shown to have high endocytic capacity [20].

Traditionally, it was thought that mouse lymphoid DCs favored a Th1 response and mouse myeloid DCs favored a Th2 response [16]. In humans, one study suggested this is reversed with lymphoid DCs generating a Th2 response and myeloid DCs generating a Th1 response [24]. This view has since been challenged and researchers have proposed that depending on the culture conditions and activation signals, DCs can acquire the capacity to stimulate a Th1 or Th2 response irrespective of lineage [24]. Thus, the same type of DC can go on to evoke more than one type of T-cell response depending on its microenvironment [24].

In 1999, Chakraborty and colleagues reported that human myeloid DCs derived from macrophages could give rise to 2 populations of DCs, one inhibitory and the other stimulatory [26]. The following year, the same group of researchers again reported that myeloid DCs, derived this time from monocytes, could give rise to two subtypes of DCs; one stimulatory and the other inhibitory [25]. They found that the stimulatory subpopulation had more co-stimulatory molecules, secreted IL-12 and stimulated naïve T-cells in a primary MLR. The inhibitory DCs had fewer co-stimulatory molecules, secreted IL-10, which is a major immunosuppressive cytokine, and did not stimulate

naïve T-cells in a primary MLR [25]. Why these results were observed is unclear. These findings enforce the extreme plasticity of DCs, and how much is still unknown about DCs.

Characterization of DCs

Previously, it was believed that there was no cell surface marker that exclusively identified DCs. Currently, there are two possible markers; DEC-205 and DC-SIGN (Dendritic cell-specific, ICAM-3 grabbing, nonintegrin,), also known as CD209 [27], [28], [29]. DEC-205 is believed to be involved in receptor mediated endocytosis by DCs [29]. DC-SIGN is proposed to bind to ICAM-3 on the surface of T-cells thus playing a role in the interaction of DCs with resting T-cells and possibly mediating the potent affect DCs have on T-cells [27]. However, until these markers are confirmed as being specific to DCs, it is still best to identify DCs by a combination of morphological, phenotypic and functional features [22].

Morphologically, DCs can be identified by the presence of numerous projections that can be hundreds of micrometers long [16]. The projections can take the form of fingerlike dendrites or veils depending on the maturational stage of the cell [16]. This is a fairly defining characteristic feature of DCs as no other blood cell exhibits this morphology [16,22].

DCs have been difficult to characterize phenotypically, partly due to the variations that occur on the cell surface during their different maturational stages, and partly because DCs are thought to share precursors to monocytes or macrophages. Thus, at certain stages, DCs may express the same markers as other cells of the immune system. DCs can be characterized both by what is present and what is absent on the cell surface.

The absence of CD3, CD19, CD56, CD14 and CD66b which are characteristic markers for T-cells, B-cells, NK cells, monocytes and granulocytes respectively, rules out cells of any of these lineages [16].

The potent antigen presenting activity of DCs is represented phenotypically by the large abundance of MHC II and MHC I molecules on the DC surface [16,30]. It has been shown that DCs express MHC-peptide complexes 10-100 times more than other APCs [31]. The interaction of DCs with T-cells is represented by the presence of various surface adhesion molecules such as CD11a (LFA-1), CD11c, CD58 (LFA-3), CD54 (ICAM-1), CD102 (ICAM-3) and CD50 (ICAM-2) [16]. The ability of mature DCs to activate T-cells is represented by the up regulation of CD80, CD86 and CD40 on the cell surface [16,30].

Two markers in particular have been identified to be preferentially expressed on DCs: CD1a and CD83 [30,32]. Although these markers are not expressed exclusively on DCs, CD1a expression is considered to be a hallmark DC marker [20,33] and the presence of CD83 is thought to be a relatively specific surface marker found on mature DCs and not commonly on other mononuclear cells (MNCs) [16,30]. CD83+ DCs were found to express the highest levels of MHC II molecules and to be the most potent stimulator cells in an allogenic MLR when compared with other leukocyte lineages [34]. In general, most researchers are in agreement that fully differentiated DCs can be identified by the combined expression of CD1a, CD40 CD54, CD80 and CD86 [19]. The presence of CD83 could further confirm that the cells are dendritic, and also to indicate that the cells have matured.

Functionally, DCs can be identified by their ability to prime T-cells [17,18]. One *in vitro* assay for testing this ability is a proliferation assay which takes advantage of the

ability of one DC being capable of stimulating the proliferation of 100-3000 T-cells [17,18]. A more in-depth discussion of the morphological, phenotypic and functional characteristics of DCs will be discussed in Chapter 4.

In Vivo Action of Dendritic Cells

The in vivo action of DCs is still incompletely understood, however the clearest and most characterized example is seen with the Langerhan cells of the skin [35].

DC Recruitment:

Immature DCs originate in the bone marrow and enter the circulation in order to traffic to non-lymphoid peripheral tissues [36]. The function of these circulating immature blood DCs remains a mystery. Some researchers have hypothesized that these blood DCs are simply in transit and serve as a source of precursor DCs whose role is to repopulate the numbers of immature DCs, known as resident DCs, residing in the peripheral tissues [36]. Equally plausible is that these circulating DCs continuously sample the endothelium for signs of inflammation. Once inflammation is detected, the cells extravasate and are recruited to the inflammatory site to pick up antigen [36]. The observation of P-selectin glycoprotein ligand (PSGL)-1 on the surface of immature blood DCs provides evidence in support of this hypothesis. (PSGL)-1 interacts with P- and E-selectins, and *in vitro* work has shown DCs interacting with P- and E-selectins on the dermal endothelium [37].

Antigen Uptake:

Immature DCs are highly effective at antigen capture [20]. They can capture antigen through different processes such as macropinocytosis, receptor-mediated

endocytosis or phagocytosis [20]. The immature DCs that have become tissue resident DCs continuously monitor the local environment for pathogens [36].

These DCs express a variety of chemokine receptors on their cell surface such as CC chemokine receptor (CCR) 1, CCR2, CCR5 and CXCR1 [36]. These receptors allow the DCs to respond to chemokines secreted during inflammation and enables the DCs to migrate to the inflammatory site by sensing a chemokine gradient [36]. Some of the inflammatory chemokines that the DCs can respond to include macrophage inflammatory protein (MIP)-1α, regulated upon activation, normal T-cell expressed and presumably secreted (RANTES) and monocyte chemoattractant protein (MCP)-1 [36]. The presence of chemokine receptors on the surface of immature DCs is not only important for migration, but also in maintaining the DC at the inflammatory site [36]. This is critical in order to maximize antigen uptake, and also to perpetuate the inflammatory response. DCs themselves are capable of secreting chemokines such as MIP-1α and MIP-2, which are known as leukocyte attractants [36].

DC Migration:

Once DCs have taken up antigen, the process of maturation is initiated and the DCs begin their journey to the lymphoid organs [20]. The down regulation of the chemokine receptors that initially attracted the DC to the inflammatory site, for example CCR5, and the up-regulation of chemokine receptors sensitive to chemokines secreted by regions such as the lymphatic vessels, for example CCR7, allows the maturing DCs to leave the non-lymphoid tissues through the lymphatic system as veiled cells [17,20]. They are termed veiled cells in reference to their cellular morphology at this point.

The veiled cells have lost most of their ability to take up antigen, and enter the draining lymph node to be directed to the paracortical area by another gradient of chemokines possibly involving CCL19 and/or CCL21 [20,38], both ligands for CCR7 [38]. The newly arrived, and now mature, DCs may themselves become a source of these two multi-functional chemokines that can serve different functions. One function is to amplify or prolong the chemotactic signal, and because these chemokines attract both mature DCs and naïve T-lymphocytes, they may also play an important role in bringing these two cell types into close proximity [20].

Antigen Processing and Presentation by Dendritic Cells:

As mentioned above, DCs are capable of presenting antigen via both MHC I and MHC II molecules to CD8+ and CD4+ T-cells respectively.

The class II MHC pathway traditionally presents endocytosed antigen to CD4+ T-cells. Immature DCs internalize and accumulate MHC II molecules into a lysosome-related intracellular compartment referred to as an MHC II rich compartment or, an MIIC [20,39]. These MHC II molecules are relatively unstable, and therefore have a short half-life [20]. The MIIC is also where immature DCs transport soluble and particulate antigen after uptake [31,40]. Once an immature cell encounters inflammatory stimuli and begins the process of maturation, several changes occur in the cell that ultimately contributes to the expression of MHC II-peptide complexes on the cell surface. Cystatin activity decreases, there is an increase in MHC II synthesis and an increase in the translocation of the MHC II-peptide complex to the cell surface where it can remain stable for many days [31,39].

Class I MHC traditionally presents cytosolic or endogenous proteins to CD8+ T-cells. The cytosolic protein to be presented is first ubiquinated and then directed to the

proteasome, a large protease complex. The proteasome cleaves the ubiquitin-protein complex into peptides that translocate to the rER and cross the rER membrane with the help of transporter associated with antigen presentation (TAP) proteins. The process of crossing of the membrane is dependent upon ATP. Once in the rER, the cleaved peptides assemble with the MHC I molecule and are shuttled out to the cell surface. DCs constitutively express di-ubiquitin, the purpose of which is unknown, but may have to do with more efficient antigen processing [20,41].

The ability of DCs to cross prime has led researchers to propose that there may be another MHC I pathway, in addition to the conventional pathway, that allows DCs to present extracellular peptides on MHC I [20]. This endogenous pathway is thought to have two routes, a TAP independent pathway and a TAP dependent pathway [42,43,44]. It is the TAP dependent pathway that is believed to be involved in immune responses against tumors, and it is thought that the manipulation of this exogenous class I pathway may have implications for cancer immunotherapy [20].

T-Cell Activation by Dendritic Cells:

It is uncertain whether the unique ability of DCs to prime naïve T-cells is due to the expression of cell surface molecules unique to the DC, or if it is due to the high expression of surface molecules involved in T-cell/DC interaction. [20]. T-cells recognize the MHC/peptide antigen complex via the TCR, which provides the immunological signal 1. The various surface adhesion molecules expressed on DCs such as CD50, CD54 and CD58 bind to their corresponding ligand present on the surface of the T-cells which promotes T-cell/DC clustering [20]. The interaction of CD40 on the surface of the DC with its ligand found on the T-cell is important in the up-regulation of the co-

stimulatory molecules B7-1 and B7-2 on the DC surface. The B-7 molecules interact with their ligand CD28 on the T-cell, which generates signal 2 [20,30]. Both signal 1 and 2 are important in order to initiate a primary immune response [20,30].

Other interactions of DC surface markers such as receptor activator of NF-κB (RANK) ligand, 4-1BB ligand and OX40 ligand generally modulate the release of cytokines and chemokines by DCs or T-cells. These can include IL-1, IL-6, IL-12, tumor necrosis factor (TNF)-α and IL-4, which are involved with increased DC survival and can help mediate T-cell proliferation [20,45,46,47,48,49,50].

DC Based Cancer Immunotherapy

The potent endocytic and antigen presenting ability of DCs make them a prime target for cancer immunotherapy. Cytokine therapy has been used in conjunction with DCs to elicit an anti-tumor response. Esche et al. observed that mice injected with lymphoma or melanoma cell lines experienced tumor regression after serial injections of Flt3 ligand (FLT3L) [51]. Flt3L, a CD34+ progenitor cell growth factor, is associated with the accumulation of DCs in tissues [49,52].

More commonly, interest in DCs revolves around the loading of tumor antigens onto DCs to elicit an anti-tumor T-cell response. For example Celluzzi and colleagues showed that mouse DCs pulsed with the strongly immunogenic ovalbumin (OVA) peptide, were able to induce an anti-tumor immune response in mice when administered as a vaccine and challenged with tumor cells transfected with OVA peptide gene [53]. Labeur et al. derived DCs from mouse bone marrow and pulsed the DCs with KLN205 lysates. KLN205 is a poorly immunogenic squamous cell carcinoma. The DCs

generated *in vitro* in the presence of GM-CSF, IL-4 and CD40 ligand exhibited protective tumor immunity [54].

DCs have also been loaded with defined peptides with known sequences, undefined acid-eluted peptides from autologous tumors and whole tumor lysates. In addition, DCs have been fused with tumors cells and transfected with retroviral vectors, adenoviral vectors and tumor cell derived RNA [16]. Nouri-Shirazi et al. pulsed immature human monocyte derived DCs with labeled and killed tumor cells. They found that the DCs were capable of taking up the killed tumor cells and cross presenting the antigen to both CD8+ and CD4+ T-cells. They were also able to show that DCs loaded with tumor antigen were capable of eliciting an anti-tumor CTL response via T-cell proliferation assays [55]. Similarly, Fujii et al. demonstrated that DCs derived from human CD34+ progenitor cells could be pulsed with irradiated leukemic cells to induce anti-leukemic CTLs [56].

The promising results of the animal and human studies described above, as well as the results of many other studies have led to several clinical trials. Hsu et al. obtained DCs from the peripheral blood of 4 different B-cell lymphoma patients. The DCs were then pulsed with either idiotype protein, produced by each tumor and isolated by cell fusion techniques, or KLH (keyhole limpet hemocyanin), an immunogenic protein that serves as a control to assess the immune response of each patient. Each patient received an infusion of autologous antigen-pulsed DCs, followed two weeks later by the subcutaneous injection of idiotype protein or KLH in saline. This vaccination strategy was repeated 4 times in each patient except for one.

Immunologically, all four patients developed cellular proliferative responses specifically against the tumor idiotype protein. Clinically, one patient achieved complete tumor regression, another achieved partial tumor regression and one patient showed no evidence of tumor cells in the bone marrow after treatment. The patient that had received three of the four planned vaccinations exhibited a minor response with some tumor regression of the peripheral lymph nodes [3].

Nestle et al. applied DC based immunotherapy to melanoma patients. DCs were derived from peripheral blood monocytes of patients. The DCs were then pulsed with HLA-binding melanoma associated antigens and administered as a vaccine into each patient at weekly intervals for the first four weeks. The fifth vaccination was received at week 6 and after that, vaccines were received monthly up to a maximum of 10 vaccinations based on the clinical response. Out of 16 patients treated in this trial, 2 patients responded with complete regression and 3 responded with partial regression [57].

Other clinical trials have been carried out in patients with prostate cancer where 9 of 33 patients partially responded when vaccinated with monocyte derived DCs pulsed with prostate tumor associated peptides [2,58,59]. Similarly, half of the renal cancer patients vaccinated with monocyte derived DCs pulsed with tumor cell lysates exhibited a partial response [2,60].

These clinical trials have demonstrated that DC based cancer immunotherapy has much potential, however the fact remains that a significant proportion of cancer patients in each trial still do not respond to treatment, and those who do respond often exhibit only partial responses. As a result, improvements to DC based cancer immunotherapy are constantly being sought. These include the use of immune adjuvants to increase the

antigen presenting ability of DCs even further, one of which is the CpG motif oligodeoxynucleotide (CpG ODN).

CpG Motif Oligodeoxynucleotide (CpG ODN)

History

A CpG ODN is a short single stranded ODN that contains one or more CpG dinucleotides in a specific base sequence context [61]. Its use as an immune adjuvant is linked indirectly to observations made more than 2 centuries ago, and the work of William B. Coley in the late 1800's.

Tumor regression after systemic bacterial infection has been documented since at least the 1700's [62]. However, it was not until the late 1800's that a large-scale effort was organized to investigate this phenomenon [62]. This effort was spear-headed by William B. Coley, an American surgeon, who became interested in this area after the death of his first cancer patient [62].

From medical records and literature available at the time, Coley observed a significant correlation between erysipelas infections and tumor regression in his sarcoma patients. Based on these observations, Coley began attempts to induce erysipelas in these patients to treat their disease [62,63]. Coley's early experiments involved local injections of live streptococcal broth cultures however, despite the presence of tumor regression and a retardation of tumor growth, the use of live broth cultures carried a serious life threatening risk [62]. As a result, Coley turned to safer methods of inducing erysipelas.

One such method involved the use of heat-killed versions of streptococci. This modification proved to be much safer, but had minimal therapeutic effects. To overcome this problem, Coley again modified his vaccine to include *Serratia marcescens* [62]. This inclusion was based on the concurrent discovery that heat-killed S*erratia* could enhance

the virulence of streptococcal cultures, and allowed for the first time, reproducible effects in a clinical setting [62].

The combination of heat killed streptococci and S*erratia* comprise what is now known as "Coley's Toxins" [62]. This concoction allowed Coley to establish a cure rate of slightly better than 10% and achieve 40% sustained clinical remissions in his treated sarcoma patients [62,64]. These are levels of success that modern day therapies have had difficulty obtaining [64]. Coley's work, as described above, may be among the earliest examples of cancer immunotherapy and as a result, has led others to bestow upon him the title, "Father of Present Day Immunotherapy." [62].

Since Coley's time, new technologies have allowed researchers to conduct more refined versions of Coley's experiments as well as to examine events at a molecular level. For example, Yamamoto et al. isolated and injected specific DNA fractions from mycobacterium bovis BCG instead of whole bacteria into the mouse [65]. They found that the DNA fractions had anti-tumor effects and that these effects correlated with the activation of NK cells and the production of interferons (IFNs) [65].

Initially, it was believed that the immuno-stimulatory and anti-tumor capabilities of the DNA fractions were due to certain "potent" palindromic sequences present in the DNA molecule [66]. Examples of these palindromic sequences included GACGTC, AGCGCT and AACGTT [66]. However, Yamamoto et al. showed that these immuno-stimulatory effects could be seen with various species of bacteria irrespective of the presence of a potent palindromic sequence [67].

DNA from five different bacterial species, as well as vertebrate and invertebrate DNA was tested for its immuno-stimulatory effects [67]. Specifically, the ability of the

DNA to activate NK cells, induce IFN and macrophage activating factor production and inhibit tumor growth was evaluated [67]. The results showed a significant immuno-stimulatory and anti-tumor effect for bacterial DNA over both vertebrate and invertebrate DNA (sea urchin, lobster, mussel, silkworm) indicating that the immuno-stimulatory and anti-tumor effects may be due to the molecular nature of bacterial DNA itself and not the presence of potent palindromic sequences [67].

Comparisons Between Vertebrate and Bacterial DNA

The inability of vertebrate DNA to generate immuno-stimulatory effects led researchers to closely examine and compare the molecular structure of both bacterial and vertebrate DNA. One of the most significant differences is a phenomenon known as "CpG suppression," where cytosine/guanine dinucleotides (CpG dinucleotides) are present at 1/4 the frequency in vertebrate DNA as compared to bacterial DNA [61]. Another difference is that CpG dinucleotides in vertebrate DNA have a cytosine that is methylated at a frequency of 80% whereas these dinucleotides are largely unmethylated in bacterial DNA [64,68].

To determine whether these differences are responsible for the immuno-stimulatory effects of bacterial DNA, researchers exploited the ability of bacterial DNA to activate B-cells. Krieg et al. synthesized ODN based on the molecular characteristics of bacterial DNA [61]. They found that synthetic ODN with one or more CpG dinucleotides could reproduce the immuno-stimulatory effect of bacterial DNA on B-cells. Conversely, synthetic ODN with no CpG dinucleotides, methylated CpG dinucleotides or methylated bacterial DNA had negligible immuno-stimulatory activity on B-cells [61]. These results indicated that the presence of unmethylated CpG

dinucleotides contributed in some degree to the immuno-stimulatory activity of bacterial DNA [61].

Similarly, Ballas et al. found that the manipulation of a synthetic ODN so that CpG dinucleotides were methylated resulted in a loss of the ability to augment NK-lytic activity [69]. They also found that the presence or absence of a potent palindromic sequence had no relevance to the immuno-stimulatory capabilities of the synthetic ODN[69].

The molecular differences between bacterial and vertebrate DNA are thought to have biological and evolutionary significance. The vertebrate immune system may recognize the absence of CpG suppression and cytosine methylation in bacterial DNA and use this as an indicator to launch an immune attack specifically on the bacteria [61]. Further supporting this idea is that various pathogens have evolved ways of evading this immune defense [70,71]. Many small DNA viruses as well as retroviruses and some parasites exhibit CpG suppression [70,71].

Development of CpG Motif Oligodeoxynucleotide

As mentioned previously, the CpG ODN is a short synthetic single stranded ODN that contains one or more CpG dinucleotides in a specific base sequence context [61]. Of importance is the presence of CpG dinucleotides as well as the bases that directly flank the CpG dinucleotides. Certain flanking bases can make one CpG ODN a more potent adjuvant [69]. In fact, another minor difference between vertebrate and bacterial DNA is that vertebrate DNA tends to have a decreased frequency of optimal flanking bases [69].

The ability of bacterial DNA and synthetic CpG ODN to activate B-cells allowed Krieg et al. to systematically determine the flanking bases for a CpG ODN to optimally stimulate the mouse immune system [61]. They found that a CpG preceded by a 5' purine-purine followed by a 3' pyrimidine-pyrimidine is most potent [12]. The 5' purines are preferably a GpA dinucleotide and the 3' pyrimidines are preferably a TpC or TpT dinucleotide [61]. A CpG ODN, identified by the label ODN 1826 illustrates these requirements.

ODN 1826: TCCATGACGTTCCTGACGTT

Unfortunately, ODN 1826 failed to have similar immuno-stimulatory effects in humans [72]. As a result, Hartmann and colleagues used the immuno-stimulatory ability of CpG ODN on human B-cells to screen for the most potent human CpG ODN in a similar fashion as done in mice [72]. They found that the requirements for a potent human CpG motif are more stringent than that required in the mouse. The CpG dinucleotides are best flanked by a 5' GpT and a 3' TpT forming the motif GTCGTT. This motif appears 3 times in the ODN and adjacent motifs are separated by TpT dinucleotides [72]. ODN 2006 fulfills these requirements.

ODN 2006: TCGTCGTTTTGTCGTTTTGTC GTT

Potency of the CpG ODN is dependent upon phosphorothioate modifications. That is, the regular phosphate bond linking bases has one of the oxygens replaced with sulfur. This makes the CpG ODN more resistant to nuclease degradation [61]. Therefore, smaller amounts of phosphorothioate modified CpG ODNs are equally potent as larger amounts of unmodified CpG ODNs [61].

Immuno-stimulatory Effects of CpG ODN

B-cells:

As previously mentioned, CpG ODNs are capable of activating B-cells both in humans and in mice. The CpG ODN is the most potent B-cell mitogen identified, and it appears to act directly on B-cells [73]. In 1995, Krieg et al. demonstrated that CpG ODNs are capable of driving over 95% of B-cells into the cell cycle as well as inducing the up-regulation of MHC II on the B-cell surface [61]. In addition to this, Davis and colleagues showed that CpG ODN up-regulates the expression of co-stimulatory molecule B7-2 on the B-cell surface. These stimulatory effects contribute to an increased antigen presenting ability of the B-cell [61,73,74]. CpG ODNs have also been shown to induce cytokine secretion from B-cells [75].

Most interestingly, CpG ODN appears to have anti-apoptotic effects on murine B-cells and the murine B lymphoma cell line WEHI-231, which undergoes spontaneous apoptosis in response to the cross linking of its antigen receptor [76,77]. At a molecular level, the anti-apoptotic effect of CpG ODN appears to prolong the activation of NF-KB, a nuclear factor that is involved in cytokine production as well as maintaining cell viability [76,77]

NK Cells:

Several experiments have demonstrated that both bacterial DNA and CpG ODN can increase the lytic activity and induce IFN-γ secretion of NK cells in mice as well as in humans [67,69]. However, CpG ODN is unable to activate highly purified NK cells [69,73]. Also, antibodies against IL-12, TNF-α and type I IFNs also abolished the ability of CpG ODN to activate NK cells [15,24]. This suggests that the action of CpG ODN on NK cells

may be indirect [73]. Chace et al. supported this idea by finding that bacterial DNA induces NK cells to secrete IFN- γ only if macrophages are present [78]. Bacterial DNA activates macrophages to secrete IL-12, which in turn induces NK cells to secrete IFN- γ. The requirement for the presence of macrophages can be replaced by introducing exogenous IL-12 [78].

In spite of this evidence, the effect of CpG ODN on NK cells does not appear to be entirely indirect. NK cells incubated with CpG ODN and IL-12 showed a higher lytic activity than IL-12 alone [61]. Thus, although CpG ODN has a significant indirect action on NK cells, it may also have a direct effect as well [61,73].

T-cells:

The effect of CpG ODN on T-cells is less characterized than those on B-cells and NK cells. In 1996, Klinman and colleagues performed an experiment that showed CpG ODN was capable of directly stimulating T-cells to secrete IL-6 and IFN- γ in the mouse [75]. However, most research in this area has suggested that CpG ODN indirectly activates T-cells, or acts as a direct co-stimulus but not a direct primary stimulus [73,79]. Klinman's results appear to be an isolated exception [73].

In the mouse, Sun et al. showed that APCs activated by CpG ODNs release IFN-I that in turn mediates the stimulation of T-cells [80]. Bendigs et al. demonstrated that CpG ODN could act as a co-stimulus for mouse T-cells by using an APC free system. They found that mouse T-cells could become activated in an APC free system provided they received a TCR mediated stimulus [79]. In contrast to the mouse system, CpG ODN failed to co-stimulate human T-cells activated with anti-CD3 or anti-T-cell receptor antibodies

[81]. In this situation, CpG ODN appeared to exert its effects on human T-cells by causing the release of IFN-I and IL-12 from peripheral mononuclear cells [81].

APCs:

As mentioned previously, CpG ODNs are capable of activating various APCs. Of these, the effects of CpG ODN on macrophages, monocytes and DCs have been best characterized. The main effects that CpG ODN appear to have on these APCs are to act on these cells directly, inducing them to secrete cytokines that either go on to activate other cells of the immune system or can induce the secretion of cytokines that favor a Th1 immune response. For example, researchers have found that CpG ODN can act directly on human monocytes to induce the release of TNF-α, IL-6 and IL-12 and up-regulate CD40, CD86 and ICAM-I [82,83]. CpG ODN can also exert its effects indirectly, as discussed earlier, or induce the secretion of cytokines from these cells that favor a Th1 immune response.

The ability of CpG ODN to directly activate DCs has received much attention recently. CpG ODN has immuno-stimulatory effects on both fetal skin-derived and bone marrow-derived dendritic cells in the mouse [72,84,85]. It appears to up-regulate co-stimulatory molecules CD80 and CD86 as well as MHC II and CD40 [84,85,86]. The up-regulation of these molecules serves as an indicator that the DCs are undergoing maturation. Thus, CpG ODN can induce the maturation of DCs [85].

CpG ODN can activate DCs to secrete Th1 cytokine IL-12 [87]. In addition to this, Sparwasser et al. found that DCs also secreted IL-6 and TNF- α in response to CpG ODN [85].

Th1 Response:

The ability of CpG ODN to induce a Th1 response with minimal toxicity is important because no adjuvant that safely induces Th1 immunity in humans has been identified [88]. For example, complete Freund's adjuvant (CFA) is a Th1 adjuvant but is associated with serious toxic side effects and has not been approved for use in humans. Alum has been approved for use in humans but it induces a Th2 response rather than a Th1 response [88].

Researchers have been able to show that CpG ODN is a more effective Th1 adjuvant than CFA based on the production of IFN-γ and IgG2a, both strong indicators of a Th1 response [68,89]. CpG ODN can induce a Th1 response in both Th1 and Th2 biased mice, and most importantly, researchers have been able to use the well characterized mouse B-cell lymphoma model, 38C13, to show that CpG ODN is able to protect mice from a tumor challenge [68,89,90].

In 1998, Liu et al. examined the immunological effects of CpG ODN on mouse DCs, as well as the ability of CpG ODN to protect from a tumor challenge. Idiotype antigen was isolated from 38C13 B cell lymphoma cells and injected subcutaneously as a vaccine with CpG ODN 3 days before the intraperitoneal innoculation of mice with viable 38C13 cells. They found that the vaccine was capable of preventing tumor growth. They also observed that CpG ODN increased the secretion of IL-12 by bone marrow-derived DCs as well as increased expression of MHC I and MHC II molecules [90].

Weiner et al. observed similar findings in 1997, where CpG ODN promoted a Th1 response and, when injected subcutaneously as a vaccine with idiotype antigen isolated from 38C13 cells, could protect from a tumor challenge. These responses were as

effective as those obtained with the Th1 immune adjuvant CFA, without the toxic side effects [68].

Mechanism of Action of CpG ODN

The molecular mechanism of action of CpG ODN is not completely understood. There is, however, general agreement that CpG ODN must be taken up into the cell in order to have an immuno-stimulatory effect [64,71,73,84,91]. Whether the DNA uptake is receptor dependent or receptor independent is unclear; no cell surface receptor specific for CpG ODN has been identified to date [91]. CpG ODNs are believed to transmit their signal through intracellular binding proteins that may be specific for the CpG motifs [73]. Factors have been identified in cytoplasmic and nuclear extracts of B-cells and monocytes, however, these were preliminary studies and efforts to identify, purify and clone these proteins are ongoing [73].

Due to the great interest of the immuno-stimulatory effects of CpG ODN on DCs, numerous studies are underway to try and identify how CpG ODN exerts its effects on DCs. A relatively recent family of receptors expressed by DCs has been identified. These receptors are called toll-like receptors (TLR), and are involved in mediating endocytosis as well as triggering innate defenses against both bacterial and fungal infections [64,36]. Of particular importance is the TLR-IL-1R pathway and TLR-9. Activation of the TLR-IL-1R pathway leads to the activation of specific kinases that ultimately result in the activation of the cell itself. The elimination of one of the molecules involved in the TLR-IL-1R pathway abolishes the ability of CpG ODN to activate cells through this pathway [64,92]. Similarly, TLR-9 mice are completely unresponsive to CpG ODN. This has lead researchers to believe that TLR-9 may be a

crucial component of a theoretical CpG ODN receptor potentially linking CpG motif recognition to the TLR-IL-1R signaling pathway [64].

Once the DNA has been taken up into the cell, it is internalized into an acidified endosomal compartment. Blocking this step with chloroquine inhibits the immuno-stimulatory activity of CpG ODN [93]. It has been hypothesized that a possible role of endosomal acidification may be to release CpG ODN into the cytoplasm so that the CpG ODN can go on to have an immuno-stimulatory effect [24]. Whatever the case, a small amount of CpG ODN ends up exiting the endosome, entering the cytoplasm and localizing to the nucleus. [91]

In the nucleus, CpG ODN exerts its effects on signaling pathways. One of the earliest detectable signaling events is the production of intracellular reactive Oxidative species (ROS) [91]. Additionally, the activation of MAPK pathways as well as the activation of various transcriptional regulatory proteins such as NF-KB and AP-1 all lead to the increase in transcription of cytokines [71,84].

Further investigation of the ability of CpG ODN to activate DCs may be important for advancing the field of DC-based cancer immunotherapy. This is the central tenet motivating our laboratory to the investigation of this idea and its further applications to cancer immunotherapy.

Purpose of this Investigation

This investigation was undertaken to achieve 3 ends. Initial efforts focused on isolating CD34+ progenitor cells in relatively pure populations from cord blood. This was done using a positive immuno-magnetic labeling method. Secondly, once CD34+ progenitor cells were reliably obtained, the ability to generate dendritic cells from CD34

progenitor cells cultured with GM-CSF, TNF- α and stem cell factor (SCF) was investigated. The development of dendritic cells was confirmed using both phenotypic and functional indicators. By staining for CD34, CD1a and CD83 expression at various time points allowed us to assess the transition between progenitor cell to immature DC and finally to mature DC. Investigating the ability of the cells to engulf antigen would indicate whether the cells were functioning as DCs. Lastly, the ability of stimulatory human CpG ODN to increase the antigen presenting ability on human dendritic cells was evaluated by looking at T-cell proliferation.

CHAPTER TWO: MATERIALS AND GENERAL METHODS

Materials

Cord blood samples were obtained from the Alberta Cord Blood Bank and the Royal Alexandra Hospital (Edmonton, Canada), according to local institutional guidelines.

The samples were collected after childbirth, via gravity feed, into blood pack units containing the anticoagulant citrate phosphate dextrose solution purchased from Baxter Healthcare Corporation, Fenwal Division (Deerfield, Illinois). All cord blood samples were diluted and processed with isolation buffer containing phosphate buffered saline and 10% fetal bovine serum both from Gibco Invitrogen Corporation (Burlington, Ontario), sterile water for irrigation and 12.5% anticoagulant sodium citrate solution from Baxter Corporation (Toronto, Ontario).

MNCs were isolated using ficoll-paque density gradient centrifugation from Amersham Pharmacia Biotech (Uppsala, Sweden). CD34+ progenitor cells were isolated from the MNC fraction of the cord blood sample using a positive CD34+ progenitor cell selection system with the corresponding magnetic particle concentrator both obtained from Dynal Biotech (Oslo, Norway).

Sheep blood in alsevers from RAMedia (Calgary, Alberta) was treated with neuraminidase from Clostridium perfringens purchased from Sigma Chemical (Oakville, Ontario). All sheep blood dilutions and washes were done in RPMI Medium 1640 made complete with penicillin-streptomycin, MEM sodium pyruvate solution 100 nM, L-glutamine and 10% fetal bovine serum all purchased from Gibco Invitrogen Corporation (Burlington, Ontario).

The last two T-cell washes were done with complete RPMI medium supplemented with male human serum type AB in place of fetal bovine serum from US Biologicals (Swampscott, Massachusetts). T-cells were cryopreserved with Cryoserv (sterile DMSO) from Research Industries Corporation (Salt Lake City, Utah).

CD34+ progenitor cells were cultured in RPMI 1640 medium made complete as described above. Specific experiments required CD34+ progenitor cells to be cultured in complete RPMI medium supplemented with male human serum type AB in place of fetal bovine serum.

Recombinant human GM-CSF at 500 U/ml from Becton Dickinson PharMingen (San Diego, California), recombinant human SCF at 50 ng/ml and recombinant human TNF-α, both purchased from Stem Cell Technologies (Vancouver, British Columbia), were added to the media.

Cells were cultured in 6 well tissue culture treated plates from Falcon Becton Dickinson and Labware (Franklin Lakes, New Jersey) or tissue culture treated flasks from Corning Incorporated (Corning, New York).

All staining was done with immuno-fluoresence buffer containing sodium azide from Sigma Chemical (Oakville, Ontario), phosphate buffered saline, and fetal bovine serum both obtained from Gibco Invitrogen Corporation (Burlington, Ontario). Cells were acquired using Becton Dickinson FACScan and Cell Quest analysis software.

IgG1κ mouse anti-human CD34 R-PE (581), CD1a R-PE (HI149), CD1a Cy-Chrome (HI149), CD16 FITC (3G8), IgG2aκ mouse anti-mouse H-2k^{d} R-PE (SF1-1.1) and controls, IgG2aκ R-PE and mouse IgG1κ FITC were purchased from Beckton Dickinson PharMingen (San Diego, California).

IgG1κ mouse anti-human CD86 (BU63) FITC and HLA-DP, DQ, DR (CR3/43) and F(ab')2 fragment of goat anti-mouse immunoglobulins R-PE were purchased from Dako Corporation (Carpinteria, California).

IgG1 mouse anti-human CD80 (MAB104) FITC, CD40 (MAB89) R-PE and control IgG1 (679.1Mc7) R-PE were purchased from Immunotech, Beckman Coulter (Mississauga, Ontario).

IgG1 mouse anti-human CD83 (HB15e) FITC was purchased from ID Labs Incorporated (London, Ontario).

General Methods

Isolation of CD34+ Progenitor Cells from Human Cord Blood

Obtaining Mononuclear Cells:

Within 48 hours after collection, whole cord blood was centrifuged at 1,450 rpm for 10 minutes. The buffy coat was collected and diluted 1:1 with (isolation buffer) IB. The diluted buffy coat suspension was layered overtop ficoll-paque and centrifuged at 1,350 rpm for 30 minutes. The mononuclear cell layer was collected, diluted 1:1 with IB and washed twice by centrifuging at 1,450 rpm for 10 minutes before counting.

Immuno-magnetic Labeling of CD34+ Progenitor Cells:

Dynabeads were washed twice by re-suspending 100µl of bead suspension in 1 ml of IB, placing the bead suspension on the magnetic particle concentrator, and aspirating off the supernatant. 4×10^7-4×10^8 mononuclear cells were re-suspended in 1 mL of IB and added to the beads. The cell/bead suspension was placed on ice and gently mixed every 5 minutes for 30 minutes. The magnetic field was then applied to select out the

CD34+ progenitor cell-bead complexes. This step was repeated three times. The extraneous cells were collected and saved on ice for T-cell isolation.

Release of CD34+ Progenitor Cells:

100 µl of DETACHabead and 100 µl of IB were added to the remaining cell-bead complexes and the suspension was incubated at 37°C with gentle vortexing ever 5 minutes for 15 minutes. The magnetic field was applied to remove the beads and the CD34+ progenitor cells were collected from the supernatant. Cells were washed twice, by centrifuging for 10 minutes at 1,200 rpm, before counting.

Culture of CD34+ Progenitor Cells:

CD34+ progenitors were resuspended in complete RPMI at a concentration no more than 5 X 10^4 cells/ml. 2.5 mls of cell suspension were placed into each well of a 6 well tissue culture treated plate. GM-CSF, TNF-α and SCF were added to each well, with SCF being withdrawn after the first five days. Cells were counted and fed cytokine on days 0, 3, 5, 7, 10, 14, 16, 18, 20, 22, 24, 28 and 30. Media volume and cytokine volume were adjusted accordingly, based on cell numbers.

<u>T-cell Isolation Using Sheep Red Blood Cells</u>

Preparation of Neuraminidase treated Sheep Red Blood Cells:

25 mls of sheep blood was diluted with 25 mls of complete RPMI and washed twice by centrifuging at 2000 rpm for 10 minutes and aspirating off the supernatant. 1 ml of the sheep red blood cell pellet was mixed with 1 unit of neuraminidase and incubated at 37°C for 1 hour with gentle mixing every 10 minutes. Neuraminidase treated sheep red blood cells were diluted with 49mls of complete RPMI supplemented with human serum type AB and washed twice by centrifuging at 2000 rpm for 10 minutes. The

neuraminidase treated sheep red blood cell pellet was diluted with 49 mls of complete RPMI and stored at 4°C until ready for use.

Mononuclear cells obtained from the extraneous cell population from the cord blood sample were suspended at a concentration of 1 X 10^7 cells/ml. A 1:2:2 ratio of MNCs, neuraminidase treated sheep red blood cells, and fetal bovine serum respectively, was gently pelleted by centrifuging at 800 rpm for 5 minutes, then incubated on ice for 1 hour. The suspension was gently re-suspended, layered overtop ficoll-paque and centrifuged at 1350 rpm for 30 minutes. The supernatant was aspirated off, and the red blood cell pellet was treated with ACK lysis buffer and immediately diluted with complete RPMI. T-cells were re-suspended and washed twice in complete RPMI by centrifuging at 1400 rpm for 10 minutes. T-cells were then counted and frozen in 90% male human type AB serum and 10% sterile DMSO.

Flow Cytometry

All steps were carried out on ice. Cells were removed from culture, counted and aliquoted into light sensitive eppendorf tubes at a concentration of 2 X 10^5 cells/tube. The cells were washed once with cold immuno-fluorescence buffer by centrifuging at 3000 rpm for 2 minutes. Antibodies were then added and diluted with immuno-fluorescence buffer to give a final concentration of 100 µl. Cells were incubated at 4°C for 30 minutes, washed 3 times with immuno-fluorescence buffer, transferred to 5 ml polystyrene round bottom tubes from Falcon Becton Dickinson and Labware (Franklin Lakes, New Jersey), and kept at 4°C in the dark until ready to acquire on the BD FACscan.

CHAPTER THREE: ISOLATING CD34+ PROGENITOR CELLS FROM HUMAN UMBILICAL CORD BLOOD

Introduction

Sources of Dendritic Cells

Research regarding dendritic cells (DCs) was initially hampered due to the inability to isolate these cells in large, homogenous quantities[18]. This obstacle has been largely overcome due to the relatively recent development of new methodologies used to isolate DCs from newly identified sources.

The most convenient source of human DCs is peripheral blood [17]. In an adult, DCs constitute approximately 1% of peripheral blood mononuclear cells (PBMCs) [19]. Due to this low percentage, the direct separation of DCs from peripheral blood is uncommon. Alternatively, DCs can be induced to differentiate from CD14+ peripheral blood monocytes that are found at a significantly higher percentage, 5-10% of PBMCs [19]. The relatively high percentage of monocytes in peripheral blood makes the derivation of DCs from monocytes a more efficient choice. Cytokines GM-CSF and IL-4 are most commonly used in combination to induce the differentiation of DCs from monocytes [17].

Human DCs can also be derived from CD34+ progenitor cells by adding a cocktail of cytokines that most commonly include granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α and stem cell factor (SCF) [17,19,52]. CD34+ progenitor cells proliferate dramatically in response to the presence of SCF in culture thus, the number of CD34+ progenitor cells capable of giving rise to DCs increases [52]. As a result, CD34+ progenitor cells rival monocytes as a source of relatively large numbers of DCs that can be conveniently obtained. In fact, CD34+ progenitor cells

may be a more attractive source for DCs than monocytes because there have been reports that monocyte derived DCs revert back into monocytes in the absence of cytokine [52]. There is also evidence that CD34+ progenitor cell derived DCs are slightly more potent at antigen presentation than monocyte derived DCs [94].

While CD34+ progenitor cells can be found in peripheral blood, they are present at a trace amount of only 0.05% making peripheral blood an unattractive source of progenitor cells [19]. Bone marrow contains higher numbers of CD34+ progenitor cells than peripheral blood. However in humans, a bone marrow aspiration is an invasive and unpleasant procedure.

Immunomagnetic Labelling: Positive and Negative Selection

The most common source of CD34+ progenitor cells is cord blood. Although it contains a mixture of both maternal and fetal blood, fetal blood contains a high percentage of CD34+ progenitor cells. In cord blood, the CD34+ progenitor cells can be found at almost six times the amount found in peripheral blood from an adult.

The CD34+ progenitors can be relatively easily isolated using immuno-magnetic labeling. The development of immuno-magnetic labeling revolutionarized the field of cell isolation. This technique involves the attachment of a magnetic particle to an antibody specific for a cell surface marker. The attachment of the magnetic particle-antibody complex to the cell surface and the subsequent application of a magnetic field allows the cells to be selected out from the entire population. Although the particle is often referred to as being magnetic, it is actually paramagnetic. The particle is of such small size that the presence of a magnetic field causes it to respond as if it were magnetic,

however the removal of the magnetic field results in the particle losing all magnetic properties [95].

There are two different approaches to cell separation using immuno-magnetic labeling, positive isolation and negative isolation. Positive isolation involves directly coupling the immuno-magnetic particle to a marker on the desired cells that can then be separated out by application of a magnetic field. The major benefit of positive isolation is that the desired cells are isolated as a very clean and pure population. However, binding of the labeling antibody to the molecule on the cell surface risks the possibility of cellular signaling and a resultant change in cellular properties.

Negative isolation involves the immuno-magnetic labeling of all cells except the desired cells. The magnetic field attracts and therefore removes all unwanted cells, leaving only the desired cells. The major benefit of using negative isolation is that the desired cells are "untouched." That is, cellular function should not be affected because no antibody binds to the cell.

Purpose of this Investigation

This study was conducted to confirm that a relatively pure and large population of CD34+ progenitor cells could be isolated from human cord blood using positive immuno-magnetic labeling. The CD34+ progenitor cells obtained by the methods carried out in this study will be used in subsequent experiments to generate DCs.

The impact of the volume of the cord blood sample and the time elapsed between sample collection and sample processing on MNC and CD34+ progenitor cell yields was assessed for the purpose of determining which cord blood samples would give optimal CD34+ progenitor cell yields.

Materials and Methods

Mononuclear cells from cord blood were isolated using ficoll-paque density gradient centrifugation as described in Chapter 2 (General Materials and Methods), and MNC counts were recorded. CD34+ progenitor cells were isolated using immuno-magnetic labeling as detailed in Chapter 2, and all cell counts were recorded.

Immediately after their isolation, between 4 X 10^4 and 2 X 10^5 CD34+ progenitor cells (depending on the final yields of CD34+ progenitor cells), were removed and stained with IgG1κ mouse anti-human CD34 R-PE (581). The presence of CD34 on the cell surface was determined using the FL-2 detector on the flow cytometer and the data was analyzed using Cell QUEST to confirm the identity of the isolated cells as CD34+ progenitor cells.

Results

<u>The Effect of Cord Blood Volume on Mononuclear Cell and CD34+ Progenitor Cell Yields</u>

A total of 45 human cord blood samples were obtained. Blood volumes ranged from 25 to 115 mls (50.5 ± 3.5 mls; Mean ± SEM), with the largest number of samples collected (n=20) falling between 25 and 44 mls.

Total mononuclear cell (MNC) counts obtained from cord blood ranged from 3.4 X 10^7 to 69.3 X 10^7 cells (21.7 X 10^7 ± 2.3 X 10^7 cells; Mean ± SEM). These cell counts were analyzed according to cord blood volume in increments of 20 mls. As expected, larger sample volumes gave rise to higher total yields of MNCs. However the relationship was not linear; volumes less than 45 mls generated approximately half as

many MNCs as volumes greater than 45 mls (14.7×10^7 cells $\pm$ 2.0×10^7 versus 27.9×10^7 cells $\pm$ 3.5×10^7; Mean $\pm$ SEM) (Figure 3-1).

The number of MNCs per milliliter (MNCs/ml) of cord blood ranged from 0.06×10^7 to 1.3×10^7 cells/ml (4.5×10^6 cells/ml $\pm$ 0.4×10^6; Mean $\pm$ SEM). When the MNCs/ml were analyzed according to volume in increments of 20mls, the results were variable, and sample volume could not be related to the amount of MNCs/ml of cord blood (Figure 3-2).

The total number of CD34+ progenitor cells isolated from human cord blood varied between 0.06×10^6 and 5.8×10^6 cells (1.1×10^6 cells $\pm$ 0.2×10^6; Mean $\pm$ SEM). Cord blood volume did not noticeably affect the total number of CD34+ progenitor cells isolated from cord blood (Figure 3-3). The number of CD34+ progenitor cells isolated per milliliter (CD34 cells/ml) of cord blood ranged from 0.1×10^4 and 23×10^4 cells/ml ($2.5 \times 10^4 \pm 0.6 \times 10^4$ cells/ml; Mean $\pm$ SEM), revealing that smaller cord blood volumes had proportionally higher numbers of CD34+ cells/ml (Figure 3-4).

<u>The Effect of the Time Elapsed between Cord Blood Collection and Cord Blood Processing on Mononuclear Cell and CD34+ Progenitor Cell Yields</u>

The time elapsed between sample collection and sample processing, ranged between 4 and 53 hrs (22.7 hrs $\pm$ 1.70; Mean $\pm$ SEM), with the majority of samples being between 12 and 23 hours. The samples were stored at either 4°C or room temperature. The disparity in storage temperatures did not affect the number of MNC and CD34+ cells obtained (data not shown).

The analysis of total MNC counts, according to the elapsed time between sample collection and processing, revealed an obvious trend with the optimal number of MNCs

being obtained between 0 and 11 hrs (34.9×10^7 cells $\pm$ 8.1×10^7; Mean $\pm$ SEM). As time between sample collection and sample processing increased, total MNC counts decreased, showing an inverse relationship (Figure 3-5). Similarly, the number of MNCs/ml of cord blood showed the same trend, with the highest value occuring between 0 to 11 hours (7.24×10^6 cells/ml $\pm$ 1.45×10^6; Mean $\pm$ SEM) (Figure 3-6).

When total CD34+ progenitor cell counts were analyzed according to the time elapsed between sample collection and sample processing, the results were variable and no noticeable effect on the counts was evident (Figure 3-7). Similarly, when CD34+ progenitor cell counts were analyzed per milliliter of cord blood, due to the large variability between samples, the time elapsed between cord blood collection and cord blood processing did not appear to significantly affect CD34+ progenitor cell counts per milliliter (Figure 3-8).

To determine the purity of the isolated population, cells from seven random samples were stained with CD34 antibody immediately after isolation and analyzed using flow cytometry. Of the seven samples, the mean percent expression of CD34 was 84.5% with an SEM of 4.5%. A representative histogram is shown in Figure 3-9.

Discussion

Cord blood rivals peripheral blood as a source of human DCs. DCs can be induced to differentiate from CD34+ progenitor cells found at relatively high proportions in cord blood. Attempts to maximize the number of CD34+ progenitor cells, in order to generate even greater numbers of DCs, have focused on the use of (SCF) and fms-like tyrosine kinase 3 ligand (FLT3L). These are both factors that act to increase the proliferation of CD34+ progenitor cells while not actually playing a role in the process of

differentiation from a progenitor cell into a DC [96]. Thus, many studies have focused on optimizing the pool of CD34+ progenitor cells after the cells have been isolated. In contrast, not many studies have examined whether the quality of the cord blood sample itself may affect the number and quality of CD34+ progenitor cells obtained.

Factors that could potentially affect the quality of the cord blood sample include whether the sample was collected immediately after birth and transection of the umbilical cord, whether the delivery was uncomplicated, the time elapsed between cord blood collection and cord blood processing, how the sample was stored before processing, and the volume of blood collected. For example, Syme et al. recently examined the effect of overnight storage of peripheral blood at room temperature (20°C) or in the fridge (4°C). They found that storage temperature at 4°C decreased the number of peripheral blood mononuclear cells obtained, altered DC morphology and resulted in DCs with decreased MHC II molecule expression on the DC surface [27].

The possibility that cord blood quality may affect the number and quality of CD34+ progenitor cells isolated is important to examine in detail. Potentially, obtaining the highest quality cord blood samples together with the use of SCF and FLT3L could give rise to maximal numbers of CD34+ progenitor cells and consequently, maximum numbers of DCs. In this study, we determined the effects of cord blood volume and the time elapsed between cord blood sample collection and sample processing on the number of MNC and CD34+ progenitor cell yields.

There was a large amount of variation between cord blood samples, not only in volume and time elapsed between cord blood collection and cord blood processing, but

also with regards to the number of MNCs and CD34+ progenitor cells isolated from each sample. In spite of this, certain conclusions could be made upon analysis of the data.

The minimum cord blood volume required for the optimal isolation of total MNCs was a sample volume over 45mls. Sample volume did not have a noticeable effect on total CD34+ progenitor cell counts. However, CD34+ cells/ml were highest when isolated from smaller sample volumes. This could be explained by the fact that smaller samples contain proportionally more fetal blood (with a higher percentage of CD34+ cells), than larger samples that may contain proportionally more maternal blood.

Even though the smaller sample volumes may give rise to more CD34+ cells/ml, probably due to the high proportion of fetal blood, the fact remains that higher total progenitor cell yields were obtained from larger sample volumes.

Cord blood processed within 0-11 hours after collection gave the highest yields of total MNCs of all time periods. Similarly, the most MNCs/ml were obtained with samples processed within this time period as well. In contrast to this, the time elapsed between cord blood collection and cord blood processing did not have a noticeable effect on total CD34+ progenitor cell yields or CD34+ cells/ml.

The fact that an increase in the time elapsed between cord blood collection and cord blood processing had a detrimental effect on MNC yields but did not have an effect on CD34+ progenitor cell yields suggests that the progenitor cells may be more resilient than other cells present in the mononuclear cell population.

The mean percent expression of CD34 at a value of 84.5% indicates that the CD34+ progenitor cells were isolated as a relatively pure population.

One problem that was encountered periodically during the isolation was contaminating red blood cells in the MNC layer after ficoll-paque density gradient centrifugation. This occurred in approximately one of every three cord blood samples collected and was completely random and impossible to predict. The cause was unknown; it could not be correlated to cord blood volume, the amount of time that had passed since sample collection or if the ficoll-paque/blood interface had been broken during the overlay. Many of these red blood cells were immature and had therefore not yet lost their nuclei. It was not possible to lyse these immature red blood cells with ACK lysis buffer. Since these red blood cells were similar in size to the MNCs, it was difficult to obtain accurate MNC counts. Therefore, samples affected by this were discarded.

In conclusion, a relatively pure population of CD34+ progenitor cells can be isolated from human cord blood using positive immuno-magnetic beads for isolation. Larger sample volumes yield greater numbers of CD34+ progenitor cells even though the smaller sample volumes contain proportionally more fetal blood. Viable CD34+ progenitor cells could be obtained from cord blood samples stored up to 48 hours prior to processing. Having determined that human cord blood could be used as a reliable source of CD34+ progenitor cells, we then went on to investigate whether these cells could be induced to proliferate and differentiate into DCs. This will be discussed in the next chapter.

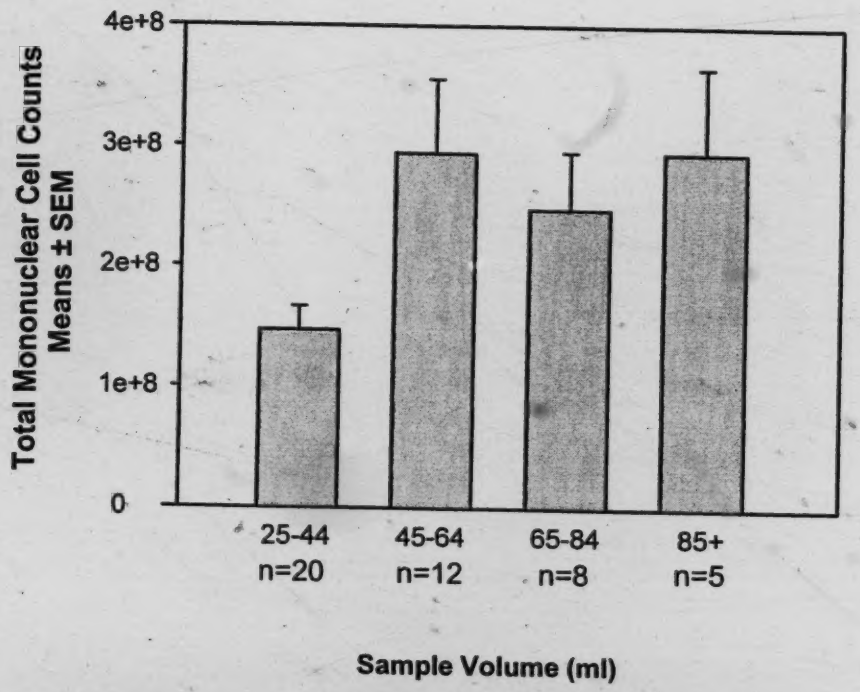

Figure 3-1. The mean counts of total number of mononuclear cells isolated from human cord blood and analyzed according to blood sample volumes. Mononuclear cells were isolated from human cord blood by ficoll-paque density gradient centrifugation.

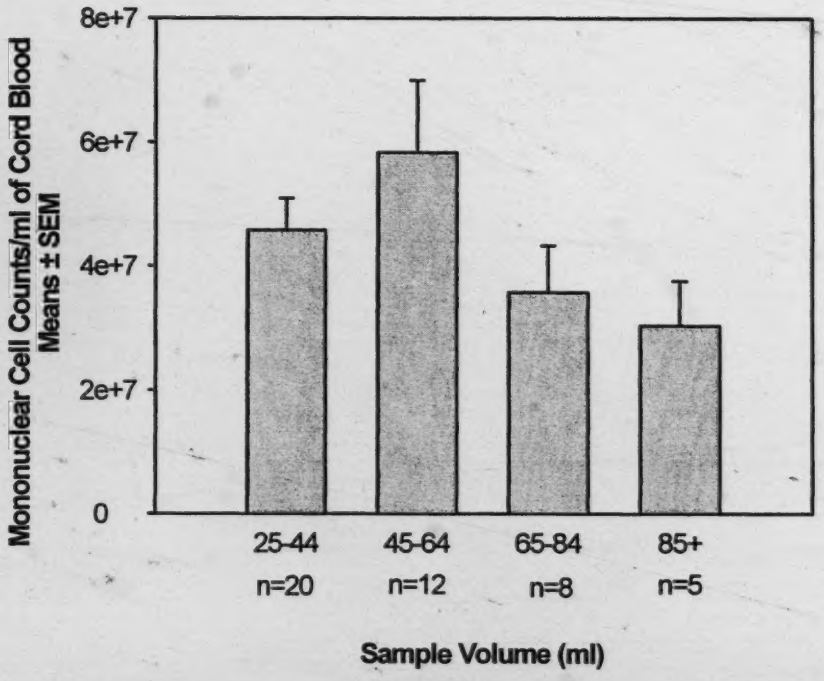

Figure 3-2. The mean counts of mononuclear cells/ml isolated from human cord blood and analyzed according to blood sample volumes. Mononuclear cells were isolated from human cord blood by ficoll-paque density gradient centrifugation.

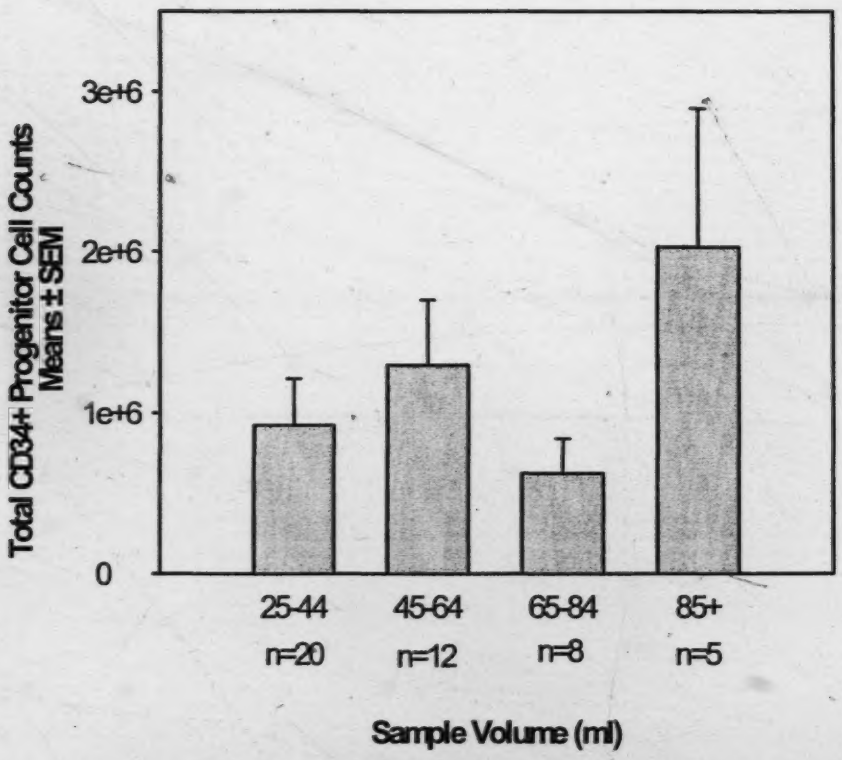

Figure 3-3. The mean counts of total CD34+ progenitor cell counts isolated from human cord blood and analyzed according to blood sample volume. CD34+ progenitor cells were isolated using immuno-magnetic labeling.

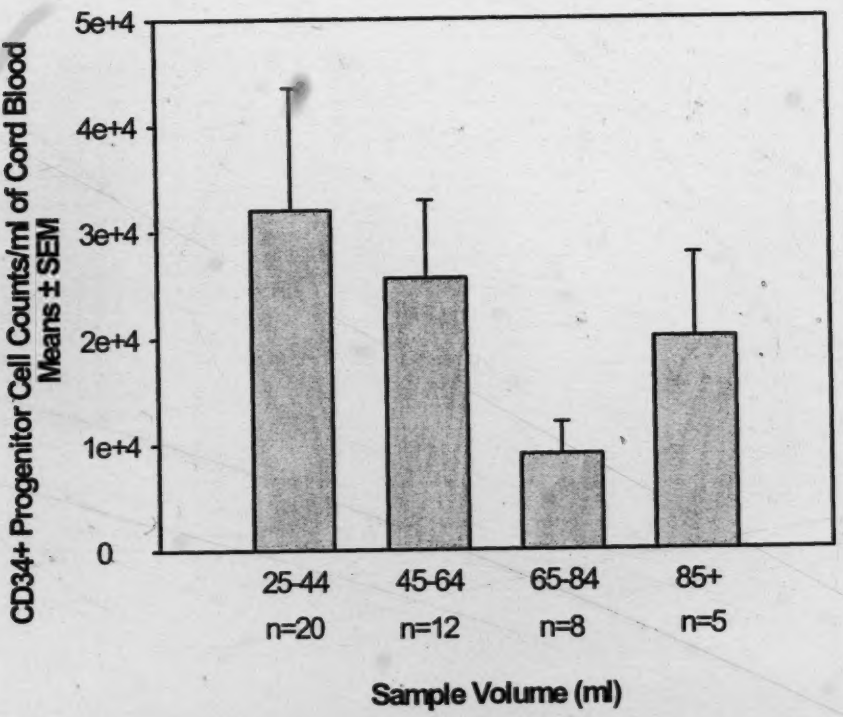

Figure 3-4. The mean counts of CD34+ progenitor cells/ml isolated from human cord blood and analyzed according to blood sample volume. CD34+ progenitor cells were isolated by immuno-magnetic labeling.

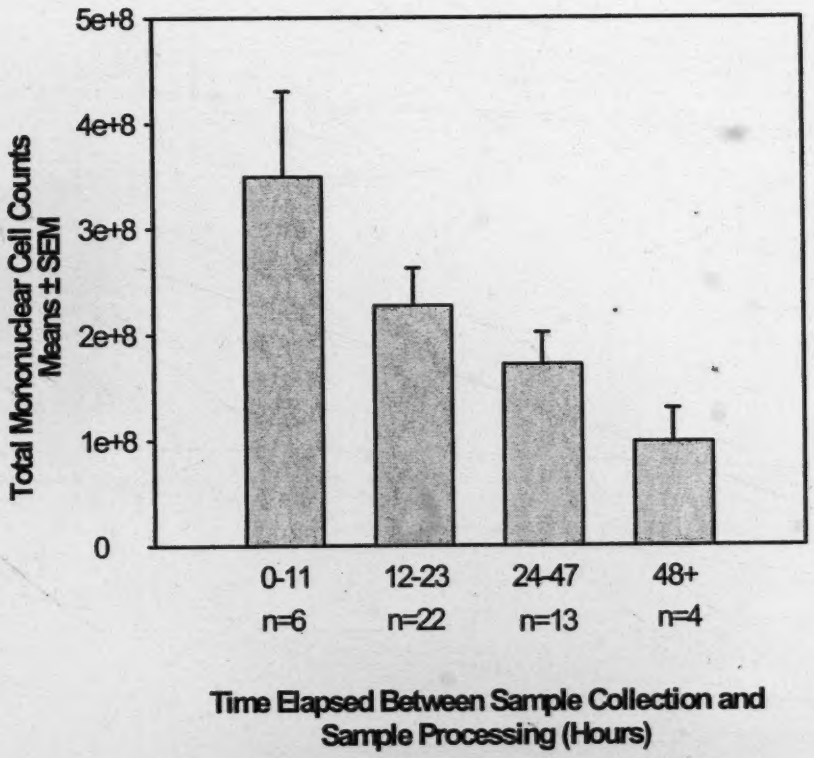

Figure 3-5. The mean counts of total mononuclear cells isolated from human cord blood and analyzed according to time elapsed between sample collection and sample processing. Mononuclear cells were isolated by ficoll-paque density gradient centrifugation.

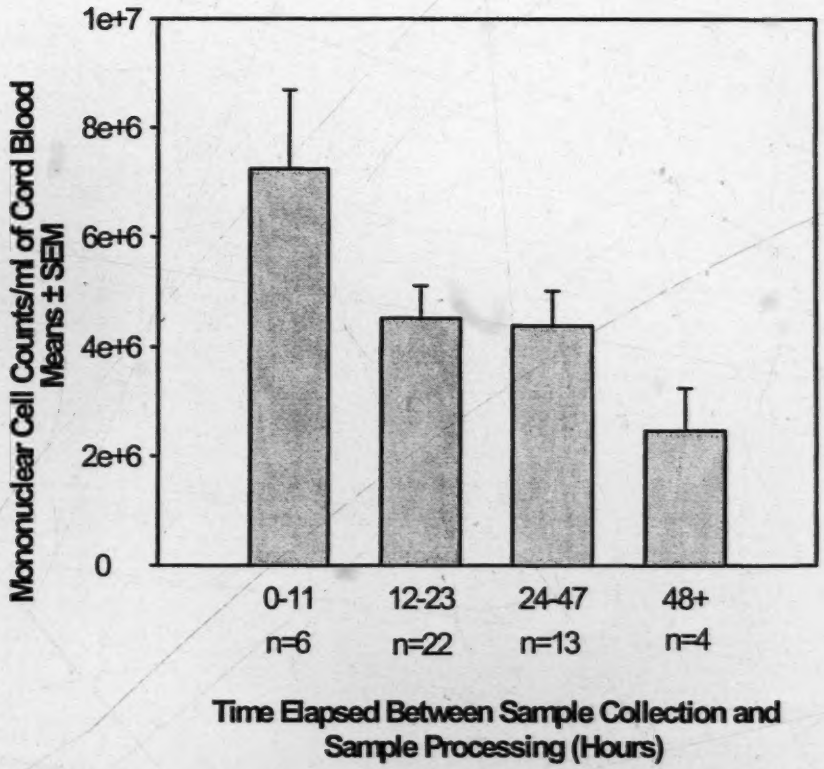

Figure 3-6. The mean counts of MNCs/ml isolated from human cord blood and analyzed according to time elapsed between sample collection and sample processing. Mononuclear cells were isolated by ficoll-paque density gradient centrifugation.

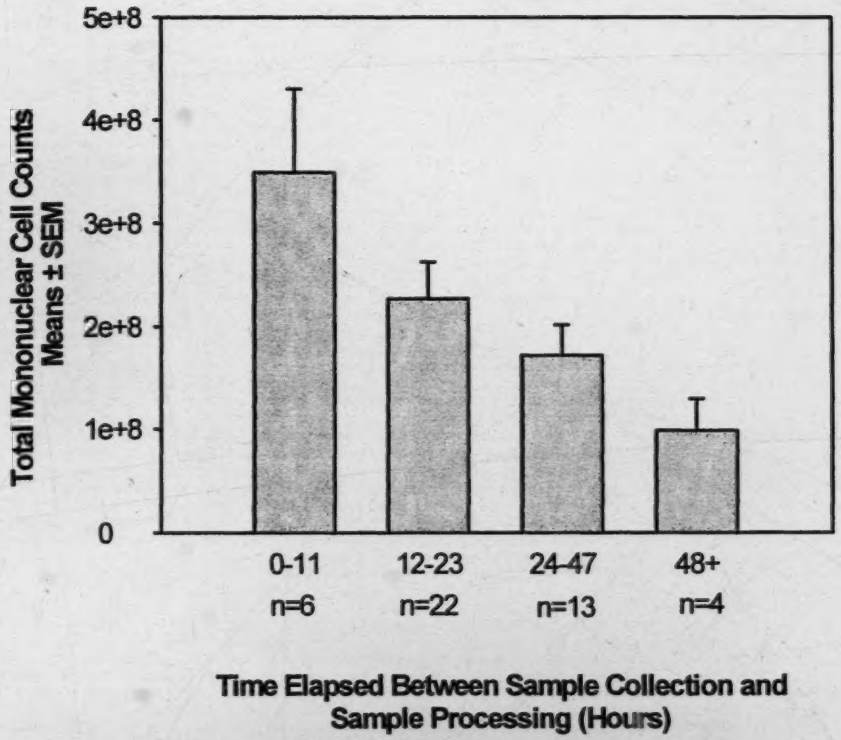

Figure 3-7. The mean counts of total CD34+ progenitor cells isolated from human cord blood and analyzed according to time elapsed between sample collection and sample processing. CD34+ progenitor cells were isolated using immuno-magnetic labeling.

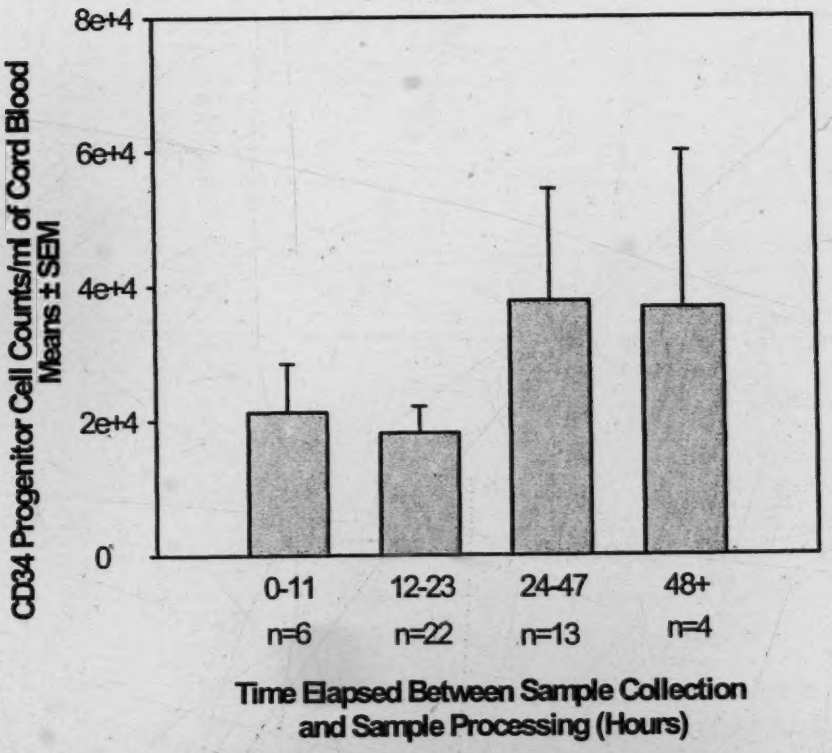

Figure 3-8. The mean counts of CD34+ progenitor cells/ml isolated from human cord blood and analyzed according to time elapsed between sample collection and sample processing. CD34+ progenitor cells were isolated using immuno-magnetic labeling.

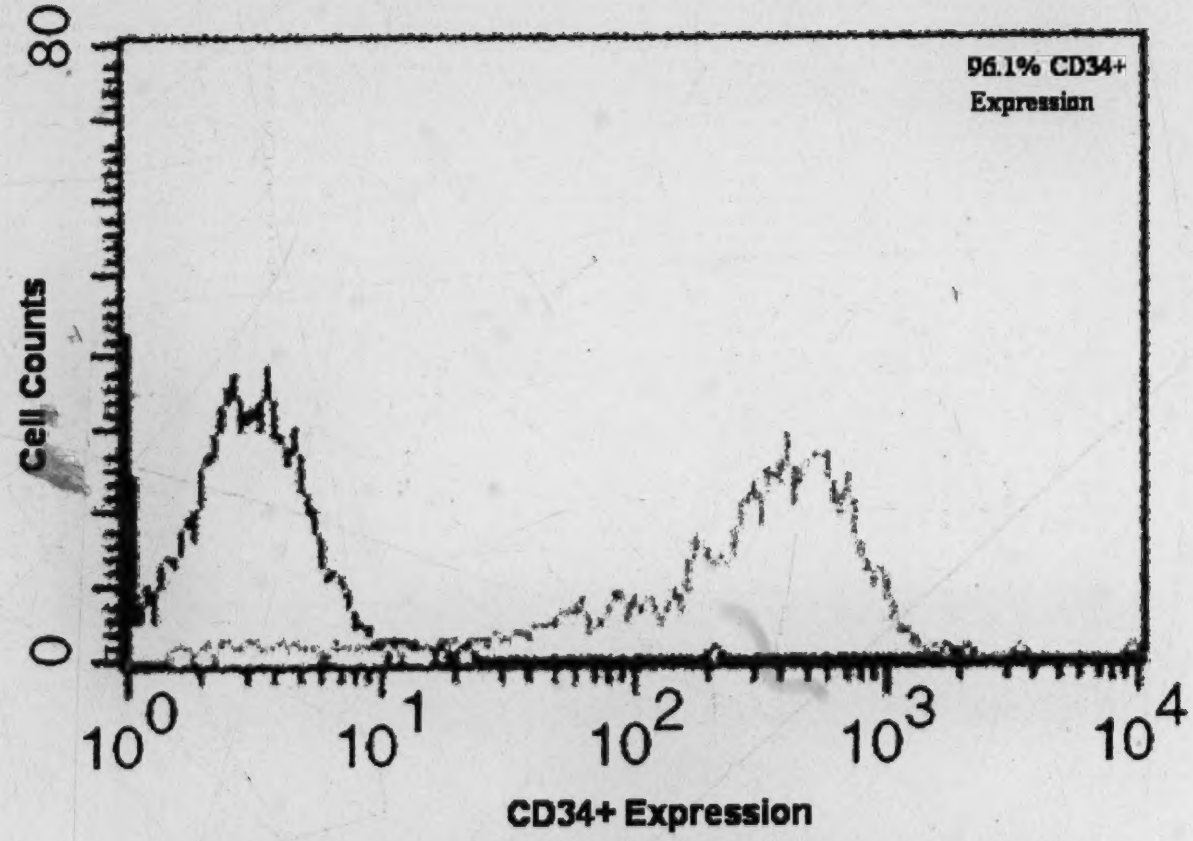

Figure 3-9. A flow cytometry histogram for a representative sample of CD34+ progenitor cells. The progenitor cells were isolated from human cord blood using immuno-magnetic labelling. The bold line represents the isotype control. The thin line represents the test condition. CD34+ expression is 94.6%.

CHAPTER FOUR: THE DEVELOPMENT AND CHARACTERIZATION OF DENDRITIC CELLS USING MORPHOLOGY, CELL SURFACE PHENOTYPE AND FUNCTION

Introduction

Development of Dendritic Cells

Human dendritic cells (DCs) can be induced to differentiate from CD34+ progenitor cells present in cord blood [33]. Positive or negative immuno-magnetic labeling techniques can be used to isolate the cord blood CD34+ progenitor cells, followed by the culture of these cells in the presence of a cytokine cocktail. Optimal cytokine cocktails allow both the proliferation of DC precursors and their differentiation into DCs [19], and commonly include granulocyte macrophage-colony stimulating factor (GM-CSF), tumor ecrosis factor (TNF)-α and stem cell factor (SCF).

The presence of SCF in the cytokine cocktail is not critical, but serves mainly to expand the population of CD34+ progenitor cells [35,39,96]. Thus, SCF does not act directly on or promote the differentiation of DCs, but instead increases the number of CD34+ progenitor cells available for DCs to differentiate from [35,96]. For this reason, SCF is only added during the first 5 days of culture [97].

While GM-CSF is a growth factor that has many functions, in terms of DC differentiation it can act alone in mice, or coupled with TNF-α or IL-4 in humans, to induce the differentiation and maturation of DCs from different sources [52]. In addition, GM-CSF has been shown to generate CD1a and MHC II expression on DCs as well as stimulate the antigen presenting ability of Langerhan cells (LCs) [17,33].

The addition of TNF-α in the cytokine cocktail is crucial to the generation of DCs from CD34+ progenitor cells in humans because it influences the commitment and enhances the maturation of CD34+ progenitor cells to DCs [33,52,98]. The combination of GM-CSF with TNF-α is key for the generation of human dendritic cells from CD34+ progenitor cells [33].

Characteristics of Dendritic Cells

Research concerning DCs was initially quite difficult due to the lack of a unique cell surface marker for identification of DCs. It is now generally accepted that DCs can be identified using a combination of morphological, phenotypic and functional characteristics [17,22].

DC morphology is unique. As a result, cellular morphology is important in identifying DCs. The term "dendritic" itself was based on the presence of numerous dendrites and projections on the cell surface [17]. Few other lymphoid cells share this morphology [2]. As DCs undergo the process of maturation, their morphology changes. For instance, during migration to the draining lymph nodes they are known as veiled DCs because of the presence of sheet-like processes on the cell surface [17]. In the lymph nodes, DCs are called interdigitating DCs due to the presence of numerous interdigitations on the cell surface [17].

There is an abundance of cell surface markers that constitute the DC surface phenotype. Previously, none of these markers were unique to the DC, but the relatively recent discovery of DEC-205 (CD209) and DC-SIGN may be possible candidates [27,28,29]. For example, there have been reports that CD1a, a principle identifying marker of human DCs, is also found on some macrophages [52]. Similarly, CD83, a cell surface marker

found on mature, activated DCs, can also be found on activated B-cells [16,99]. As a result, a constellation of cell surface markers must be taken into consideration when identifying DCs [17,35]. The most common markers used to identify human DCs are CD1a, CD40, CD54 (ICAM-1), CD58, CD80, CD86 and CD83 [17,35]. Additionally, DCs also express very high levels of MHC I and MHC II [16,30]. Many of these cell surface molecules reflect the ability of DCs to interact with and stimulate T-cells.

As CD34+ progenitor cells begin the process of differentiation to DCs, several changes occur. CD34+ expression is down-regulated, which coincides with an up-regulation of CD1a expression [98]. CD1a is a hallmark DC marker and CD1a+ DCs are reported to constitute approximately 20-50% of cells after 12 days of culture of CD34+ progenitor cells [52,98]. The onset of CD1a expression signals the transition from progenitor cell to immature DC. Due to their potent ability to take up and process antigen, immature DCs are of particular interest for studies involving loading DCs with antigen such as tumor antigen. One of the best-characterized examples of immature DCs is the Langerhan cells of the skin [35].

Observations from other laboratories have reported that after approximately 12 to 14 days in culture, CD83 expression begins to appear [35]. This is an observation that has been confirmed by our laboratory as well. CD83 is a marker associated with mature DCs [16,30] thus, the appearance of CD83 signals the transition between immature and mature DCs. Mature DCs have lost the ability to take-up antigen but are specialized in presenting antigen to naïve T-cells [20].

The functional ability of DCs is almost as unique as the cellular morphology. DCs are the most potent APCs identified, and they are the only APC capable of eliciting

a primary immune response. That is, they can stimulate naïve T-cells and cause their expansion [21]. DCs exhibit more endocytic activity than any other cell type [21].

Matzinger et al. were able to demonstrate that resting mouse DCs can endocytose both necrotic and apoptotic cellular fragments using the following engulfment study [1]. In their experiment, BALB/c (H-2^d) fibroblasts were stained with the intracellular dye, CFDA SE. This compound is initially non-fluorescent, and diffuses passively into live cells only. Intracellular esterases within the cell act on CFDA SE, and cleave its acetate groups resulting in CFSE, which has fluorescent properties. CFSE is retained within the cell due to its ability to interact with intracellular amines and it emits light of wavelengths between 505-545 nm (green fluorescence), which is easily detectable by the FL-1 detector on the flow cytometer. When cell division occurs, the dye is divided among the daughter cells, and consequently, the intensity of the dye diminishes. After staining with CFDA SE, the fluorescing mouse fibroblasts were either made into necrotic fragments through multiple freeze/thaws, or apoptotic fragments by treating with apoptotic agents, ceramide and mytomycin c. H-2^b mouse DCs were then co-cultured with the fluorescing necrotic H-2^d fibroblast fragments to promote engulfment, before staining with monoclonal antibodies.

Triple staining with a membrane impermeable H-2^d antibody after engulfment allowed Matzinger and colleagues to differentiate between two possible scenarios: the engulfment of the fluorescing necrotic fragments by the mouse DCs or the adhesion of the fluorescing necrotic fragments to the outside surface of the mouse DCs. Mouse DCs were identified using antibodies against CD11c (red), H-2^d+ mouse fibroblasts and necrotic mouse fibroblast fragments were identified by the presence of CFSE (green

fluorescence) as well as by membrane impermeable antibodies against H-2^d (orange). If the mouse DCs had engulfed the fluorescent fragments, the presence of CD11c (red) and CFSE (green) would be detected by the flow cytometer, however H-2^d (orange) would not be present because this antibody would not be able to cross the mouse DC membrane to stain the engulfed fragments. If the mouse DCs had fluorescent fragments adherent to the outside cell surface, the presence of CD11c (red) and CFSE (green) would be present, as well as H-2^d (orange) since the H-2^d antibody from binding to the necrotic mouse fibroblast fragments in these circumstances (Figure 4-1).

Matzinger et al. showed that both apoptotic and necrotic fibroblasts were equally likely to be taken up by DCs [1]. However, unlike the necrotic fibroblasts, apoptotic fibroblasts were unable to activate DCs in vitro and induce an in vivo primary immune response. In addition to demonstrating the endocytic activity of mouse DCs, they showed that mouse DCs are sensitive to mechanical stimulation such as manual pipetting. They responded to such stimulation by transitioning from a resting DC to an activated DC. That is, they up-regulated B7-1, B7-2, MHC II and CD40 [1].

Purpose of this investigation

The purpose of this investigation was to generate DCs from the human cord blood CD34+ progenitor cells isolated as outlined in Chapter 2 using GM-CSF, TNF-α and SCF, and to confirm that the differentiated cells were dendritic cells by assessing cell morphology, cell surface phenotype and functional ability.

The cell morphology of the CD34+ progenitor cell cultures was visually monitored on a daily basis. At sequential time points, cell surface phenotype was assessed by staining with monoclonal antibodies directed against CD34, CD1a and

CD83. The presence of these markers on the DC surface was determined using flow cytometry. These results also served to indicate the temporal progression from progenitor cell to immature DC to mature DC.

Functional ability was evaluated using immature human DCs, identified using cell surface phenotype, and assessing their ability to take up necrotic cellular fragments. The experimental design for assessing the ability of immature DCs to take-up necrotic cellular fragments was based on Matzinger's experiment as described above, with a few modifications. Immature CD1a+ human DCs derived from CD34+ progenitor cells were used in place of the mouse H-2^b DCs, and the mouse lymphoma cell line, LT210, replaced the BALB/c(H-2^d) fibroblasts. This mouse lymphoma cell line was used instead of primary human tumor cell lines due to its rapid proliferation, which is a luxury not often present in the culture of primary human tumor cell lines, and because the LT210 cells could be easily identified by staining for the cell surface marker H-$2K^d$. The LT210 cells express H-$2K^d$, another mouse surface marker analogous to MHC I in humans. Cell surface markers for primary human tumor cell lines are often difficult to obtain. The LT210 cells were stained with CFDA SE, made into necrotic fragments and co-cultured with immature human DCs. To determine which DCs had taken up the necrotic fragments, staining with monoclonal antibodies towards CD1a and H-$2K^d$ was done.

Materials and Methods

Examination of Dendritic Cell Morphology and Cell Surface Phenotype:

CD34+ progenitor cells were isolated from cord blood and cultured as described in Chapter Two. Briefly, MNCs were isolated from cord blood using ficoll-paque density gradient centrifugation and CD34+ progenitor cells were obtained via direct immuno-

magnetic labeling of MNCs. The progenitor cells were then placed into culture with a cytokine cocktail containing GM-CSF, TNF-α and SCF.

Cell cultures were counted on days 0, 7, 10, 14, 20, 24 and 30, and aliquots of approximately 1 X 10^6 cells were removed at these time points and stained with IgG1κ mouse anti-human CD34 R-PE (581), CD1a R-PE (HI149) and CD83 FITC (HB15e). In some cases, cells were stained with both CD1a and IgG1k mouse anti-human CD16 FITC (3G8) to confirm the absence of macrophages. Cells were acquired on the flow cytometer using the FL-1 and FL-2 detectors, and data was analyzed using Cell Quest. Methods for flow cytometry staining are detailed in Chapter 2.

Examination of Antigen Uptake by DCs

Preparation of Stained, Necrotic LT210 Cells:

LT210 cells were kindly provided by Dr. Kevin Kane (Department of Medical Microbiology and Immunology, University of Alberta) and cultured as non-adherent cells in tissue culture treated flasks from Corning Incorporated (Corning, New York) with complete RPMI. Due to the rapid proliferation of these cells, the culture medium was replaced every 2 to 3 days.

1 X 10^6 non-adherent LT210 cells were removed from culture, washed once in complete RPMI and stained with the Vybrant™ CFDA SE Cell Tracer Kit purchased from Molecular Probes (Eugene, Oregon). Briefly, the staining procedure involved re-suspending the cells in 1ml of 10 μM CFDA SE dye, pre-warmed to 37°C, then transferring the cell suspension to a 6 well tissue culture treated plate and incubating at 37°C for 10 minutes.

Following the incubation, the cells were collected into a micro centrifuge tube, washed twice and irradiated with 3000 rads in order to arrest their rapid proliferation and the subsequent distribution of the dye to daughter cells resulting in the diminished intensity of the CFDA SE dye. The irradiation should not affect the cell surface expression of H-2K^d. Using a slurry of dry ice and 70% ethanol, necrotic fragments were prepared from the cells using a series of repeat freeze/thaws. Briefly, cells were placed in the cold slurry mixture for 5 minutes then thawed at 37°C for 5 minutes. This procedure was repeated 3 times. Staining of LT210 cells was confirmed under a fluorescent microscope to ensure CFDA SE uptake and cleavage to form the fluorescent end product, CFSE.

Co-culture of CD1a+ Cells and Stained Necrotic LT210 Cellular Fragments:

At day 10, 1 X 10^6 cells were removed from the CD34 progenitor cell cultures, prepared from human cord blood as detailed in Chapter 2. These cells were washed once and resuspended in 29mls of complete RPMI before the addition of 1 ml of LT210 fragments made from 1 X 10^6 LT210 cells. 500 U/ml of GM-CSF and 50 U/ml of TNF-α were added to the culture and the cells were incubated for 24 hours at 37°C, 5% CO_2.

Flow Cytometry:

After 24 hours, the co-cultured cells were double stained with IgG1κ mouse anti-human CD1a Cy-Chrome (HI149) and IgG2aκ mouse anti mouse H-2k^d R-PE (SF1-1.1) antibody according to methods described earlier. Cells were acquired using the FL-1, FL-2 and FL-3 detector on the flow cytometer, and analyzed using 3-color analysis.

Results

Examination of Dendritic Cell Morphology and Cell Surface Phenotype

As CD34+ progenitor cells remained in culture, transformations in shape began to occur. As early as the first day of culture, tiny blebs could be seen arising from the cell surface. These cells were non-adherent. By day 5 most cells were elongated and some were beginning to develop projections. The formation of small cell clusters was also evident at this point. By day 7, cellular projections were more evident and non-adherent cell colonies were beginning to form. These cell colonies increased in size and developed more defined cellular projections by day 10. By day 14 of culture, many of these colonies had become adherent and the cells in the colonies had even more projections (Figure 4-2).

The proliferation of CD34+ progenitor cells in culture was dramatic. The initial number of isolated cells placed into culture was 5.1×10^5 cells $\pm$ 2.3×10^5 (Mean $\pm$ SEM). Numbers increased steadily so that by day 14, the cell numbers had increased more than tenfold (7.3×10^6 cells $\pm$ 2.8×10^6; Mean $\pm$ SEM). By day 20, the cell numbers dropped off dramatically (Figure 4-3), presumably due to cell death.

CD34+ progenitor cells typically began to increase in size and granularity after approximately 5 days in culture. By day 20, this process appeared to have stabilized. Gating around the larger more granular population of cells revealed that this population contained the majority of CD1a+ cells. As a result, analysis after day 10 was focused on the cells within this gate.

On day 0, immediately after CD34+ progenitor cell isolation, CD34+ expression was 84.5% ± 4.6 (Mean ± SEM), while CD1a and CD83 expression remained low (4.3% ± 1.0 and 1.4% ± 0.4 respectively; Mean ± SEM). By day 10, the expression of CD34+ had virtually disappeared (1.1% ± 0.8; Mean ± SEM) however this decrease was accompanied by an increase in CD1a expression, which ultimately peaked at day 20 (89.2% ± 1.5; Mean ± SEM). CD83 expression was first noticeably up-regulated on day 14 and, in a similar fashion as CD1a, reached a peak at Day 24 (28.7% ± 6.50; Mean ± SEM). These data are shown in representative histograms detecting the presence of CD34, CD1a and CD83 in Figure 4-4, as well as graphically represented in Figure 4-5.

The double staining of cells from three independent CD34+ progenitor cell cultures at day 10 with CD1a and CD16 revealed little to no presence of CD16 on the surface of CD1a+ cells present within the cultures (0.2% ± 0.2; Mean ± SEM) as well as negligible amounts of CD16 expression in the total cell culture (data not shown). Therefore, we were confident that the CD1a expression reflected the presence of DCs, not macrophages. On one occasion, staining for B7-1, B7-2 CD40 and MHC II revealed that CD1a+ cells were also positive for B7-1 (84.2%), B7-2 (77.8%), CD40 (91.8%) and MHC II (94.5%).

In addition to the findings mentioned above, the concentration of cytokine per cell was found to be critical. In overcrowded conditions, or when the number of cells was so great that the amount of cytokine per cell was below 0.005 units of GM-CSF/cell, no increase in granularity or size occurred, and the up-regulation of CD1a and CD83 was absent (data not shown). If the number of cells present in culture was such that the amount of GM-CSF/cell was kept greater than or equal to 0.005 units of GM-CSF/cell,

changes in granularity and size occurred as well as an increase in CD1a and CD83 expression.

Examination of Antigen Uptake by Dendritic Cells

For three random samples, the CD1a expression of the cells used in the co-culture experiments with necrotic LT210 fragments after 24 hours of co-culture ranged between 56.7% and 92.0% (74.7% ± 5.7; Mean ± SEM). Of these CD1a+ cells, a mean of 33.6% ± 5.9 of cells co-expressed CFSE (green fluorescence) but not H-2K^d. That is, approximately 33% of the CD1a+ cells had actually engulfed the fluorescent necrotic fragments. The percentage of the CD1a+ cells that were simultaneously positive for both CFSE and H-2K^d expression was approximately 2.6% ± 1.7%. These are CD1a+ cells that have fluorescent fragments attached to the outside cell surface. Therefore we conclude that approximately 30% of the cells had engulfed fragments. A representative scatter plot is shown in Figure 4-6.

Discussion

As mentioned previously, DCs are best identified using a combination of morphological, phenotypic and functional characteristics. For example, Fujii et al. investigated the ability of CD34+ progenitor cell derived DC clusters to present tumor antigen [56]. Their observations included that the developing DCs assumed a dendritic shape, were positive for CD1a, CD4, CD54, CD86 and MHC II and were capable of engulfing latex particles and stimulating an MLR [56]. These are all characteristics associated with DCs. Similarly, in this study we investigate the presence of DCs using morphology, cell surface phenotype and cell function.

Rosenzwajg et al., and Caux et al. indicate that the morphology of DCs derived from CD34 progenitor cells using GM-CSF and TNF-α exhibit membrane projections[32,33]. In addition to these observations Zheng et al., and Sato et al. both reported the formation of cell clusters consisting of dendritic–like cells [100,101]. From our own observations, the morphology of the cells differentiating from CD34+ progenitor cells coincides with the morphological descriptions of DCs made by other investigators.

Monitoring the mean percent expression of CD34, CD1a and CD83 on the surface of the differentiating cells contributes information about the cell surface phenotype of the cells as well as their maturational stage. The mean percent expression of the cell surface markers was used to express results instead of the mean fluorescence intensity because it was assumed that it was more important to simply detect the presence of the markers on the cells' surface rather than showing how strongly the markers were being expressed on the cells. However, the pattern of mean fluorescence intensity was similar to that of the percent expression (data not shown) with the exception that the peak of CD1a and CD83 occurred earlier on day 14 versus day 20. Despite the difference, similar conclusions could be made from both sets of data.

Herbst et al. found that CD34+ expression disappeared by day 7 on CD34+ peripheral blood progenitor cells cultured in IL-3, IL-6, SCF, IL-4 and GM-CSF [94]. Similarly, Rosenzwajg et al. have shown that CD34+ expression was less than 10% by day 5 of culture in GM-CSF, TNF-α and SCF. These are consistent with our findings that CD34+ expression is no longer apparent by day 10 of culture. Rosenzwajg et al. report that CD1a is expressed on approximately 75% of gated cells by day 12 [32]. CD1a

expression was not assessed specifically on day 12 in our studies however, by day 14, over 80% of gated cells were expressing CD1a.

The concurrent loss of CD34 expression and up-regulation of CD1a by day 7 serves as an indicator that CD34+ progenitor cells are beginning the process of differentiation into CD1a+ DCs. This is further reinforced by the up regulation of CD83, a mature DC marker, by day 14. The expression of both CD1a and CD83 strongly indicates that the developing cells are DCs. To solidify the conclusion that the CD1a+ and CD83+ cells were DCs, double staining was done with CD1a and CD16 monoclonal antibodies. Only a negligible amount of CD1a and CD16 expression was present ensuring that the CD1a expression was not from macrophages.

In this study, we used the presence or absence of CD34, CD1a and CD83 cell surface expression at different time points as an indicator of the cells maturational stage. By doing this, we were able to determine the window of time that DCs exist in an immature state, and we were able to confirm this by demonstrating that these cells are capable of taking up antigen.

The loss of CD34 expression by day 10, which coincides with an up regulation of CD1a, signals the transition from CD34+ progenitor cell status to CD1a+ immature DC. This is further reinforced by the absence of CD83 expression until day 14. By day 14, the appearance of CD83 indicates the onset of the transition between immature DC to mature DC. Thus, the window of time where the majority of the DCs are immature and therefore capable of taking up antigen is approximately between day 10 and day 14.

The functional ability of the immature DCs was determined using an engulfment study based on Matzinger's et al experiments [1]. The results of our study showed that a

percentage of CD1a+ cells (approximately 33.6% ± 5.9; Mean ± SEM), cultured for 10 days were capable of antigen uptake. This result is consistent with the engulfment study conducted by Fujii et al. where they demonstrated that approximately 25% of cells actively ingested latex particles [56].

Although antigen uptake by CD1a+ cells was apparent, the percentage of cells that had taken up antigen was low compared with the total percentage of CD1a+ cells present. The most likely cause for this result was the conditions present when co-culturing the necrotic cellular fragments with the cells from the CD34+ progenitor cell cultures. The large 30ml total volume of the co-culture likely decreased the amount of interaction between the CD1a+ cells and the cellular fragments. Therefore, more antigen uptake may occur if the co-culture volume was significantly smaller and carried out in a 96 well plate.

These results could likely have been improved by further characterizing the fragment population used. There may be an optimal fragment concentration that should be fed to the CD1a+ cells or an optimal fragment size. However, this is difficult to control with the freeze/thaw method of generating necrotic fragments. Also, manipulating the amount of time that the CD1a+ cells are exposed to the necrotic cellular fragments for cellular uptake could have an effect. Lastly, it has been proposed that DCs may capture killed tumor cells through their expression of specific receptors such as vitronectin, thrombospondin and receptor CD36 [55]. However, at a given time point, there is great heterogeneity in DC surface expression [55], meaning that the expression of these receptors varies considerably and may affect the ability of DCs to take up antigen [55]. This receptor heterogeneity may also contribute to the relatively low proportion of

CD1a+ cells capable of antigen uptake. In addition, the specific ligands for these receptors may not be present on the mouse lymphoma cell line that was used in our experiments.

We are confident that CD1a+ immature DCs are being generated from cord blood CD34+ progenitor cells as indicated by cell morphology, cell surface phenotype and functional ability which is consistent with that seen by other researchers in the field. These cells have numerous dendrites and projections on the cell surface and form the characterisitic cell clusters. They express CD1a and CD83, markers associated with immature and mature DCs. Based on the temporal pattern of CD1a and CD83 surface regulation, immature DCs were identified between day 10 and day 14 of culture, and these cells were capable of taking up necrotic cellular fragments albeit low.

Although many researchers have reported that immature DCs are capable of taking up antigen, few researchers have investigated the time frame surrounding the development of immature DCs from CD34+ progenitor cells and the subsequent differentiation into mature DCs. In this study, by assessing the up regulation and down regulation of cell surface markers, CD34, CD1a and CD83, we were able to determine when immature DCs were present, and we were able to confirm their immature state by showing that these cells were capable of antigen uptake.

DCs have garnered much attention due to their potential therapeutic application in treating diseases such as cancer via their ability to stimulate strong immune responses. One way to further enhance the therapeutic effect of DCs is to use immune adjuvants. Having confirmed that we have a reproducible source of functional immature DCs, in the

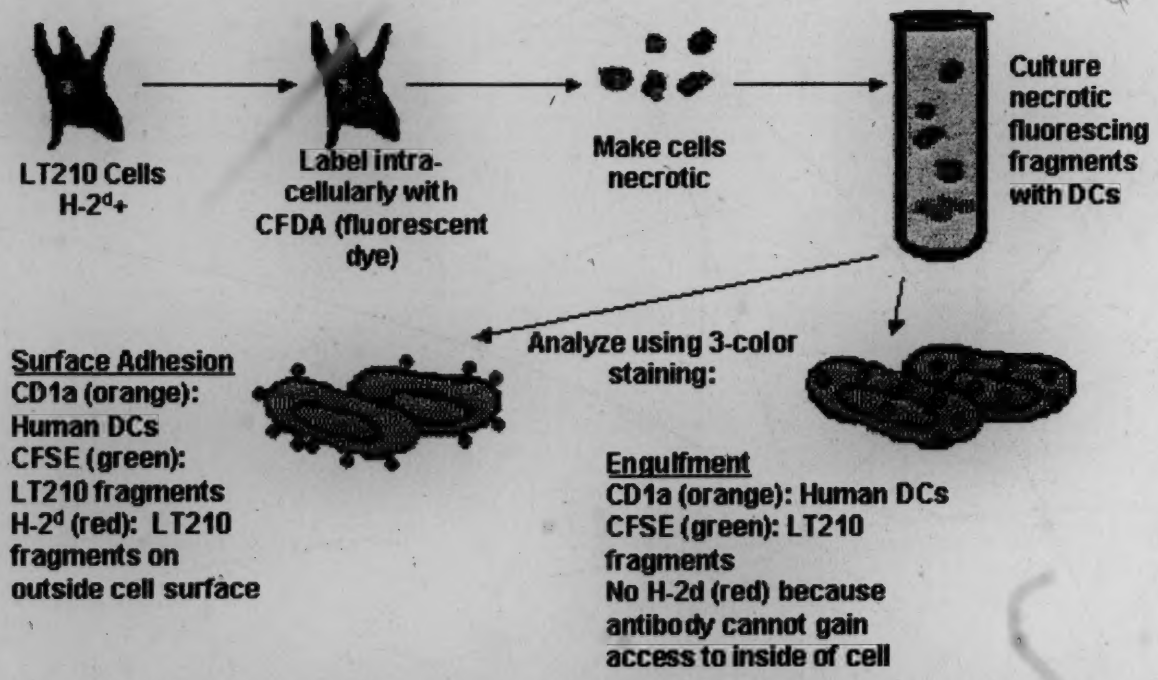
LT210 Cells
H-2d+
Label intra-cellularly with CFDA (fluorescent dye)
Make cells necrotic
Culture necrotic fluorescing fragments with DCs
Analyze using 3-color staining:
Surface Adhesion
CD1a (orange): Human DCs
CFSE (green): LT210 fragments
H-2d (red): LT210 fragments on outside cell surface
Engulfment
CD1a (orange): Human DCs
CFSE (green): LT210 fragments
No H-2d (red) because antibody cannot gain access to inside of cell

Figure 4-1. Experimental Design for Assessment of Antigen Uptake by DCs. Methodology is based on Matzinger's experiment . Briefly, LT210 cells are labeled with intracellular CFSE, made necrotic and then fed to immature DCs. DCs are then analyzed using 3-color staining. Cells positive for orange, red and green have necrotic fragments attached to the outside cell surface. Cells positive for only orange and green have taken up necrotic fragments.

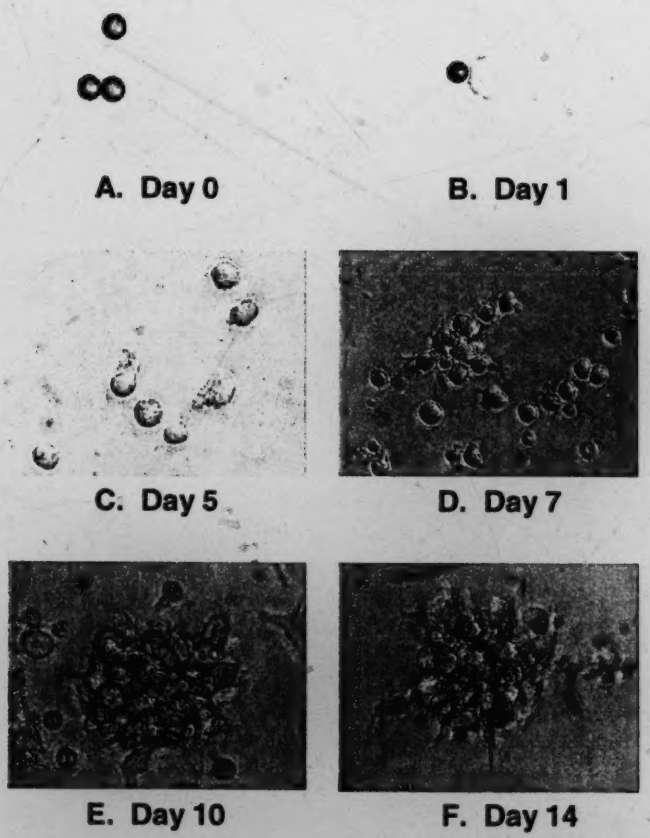

Figure 4-2. CD34+ progenitor cells cultured between 0 and 14 days in media supplemented with GM-CSF and TNF-α. SCF was added for the first 5 days only. Photos (A) and (B) contain CD34+ progenitor cells attached to Dynabeads.

next chapter we will investigate the effects that immune adjuvant CpG ODN has on the antigen presenting ability of these DCs.

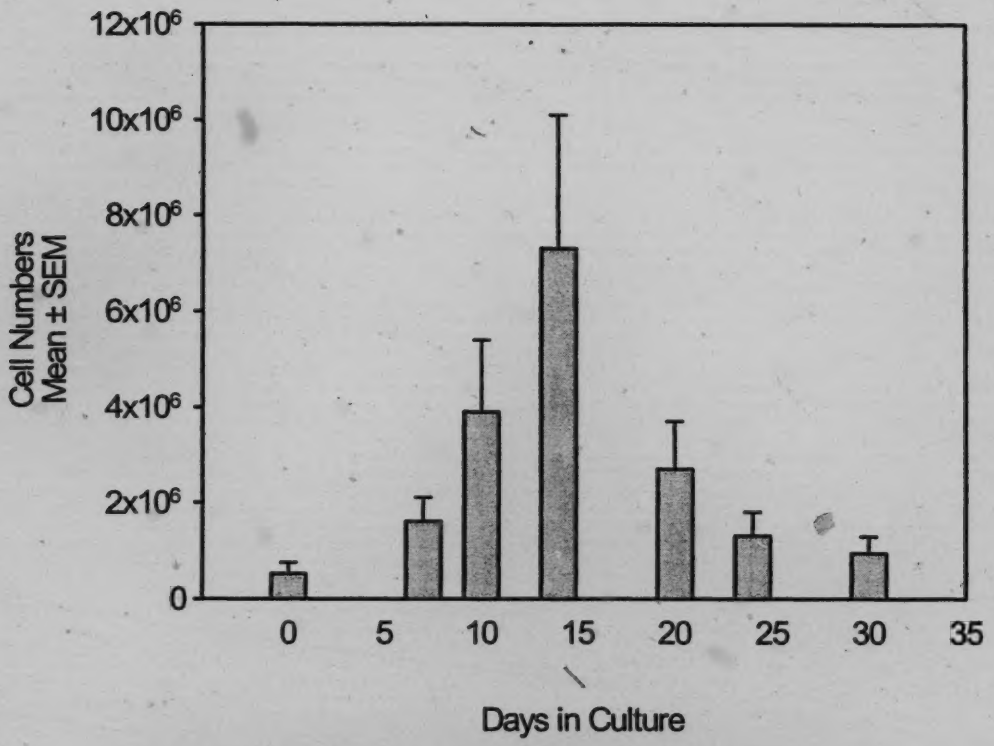

Figure 4-3. The mean cell numbers derived from CD34+ progenitor cells isolated from human cord blood and cultured for 30 days (n=7). GM-CSF concentration and TNF-α concentration were present in the culture throughout the 30 days while SCF was present for the first 5 days of culture only.

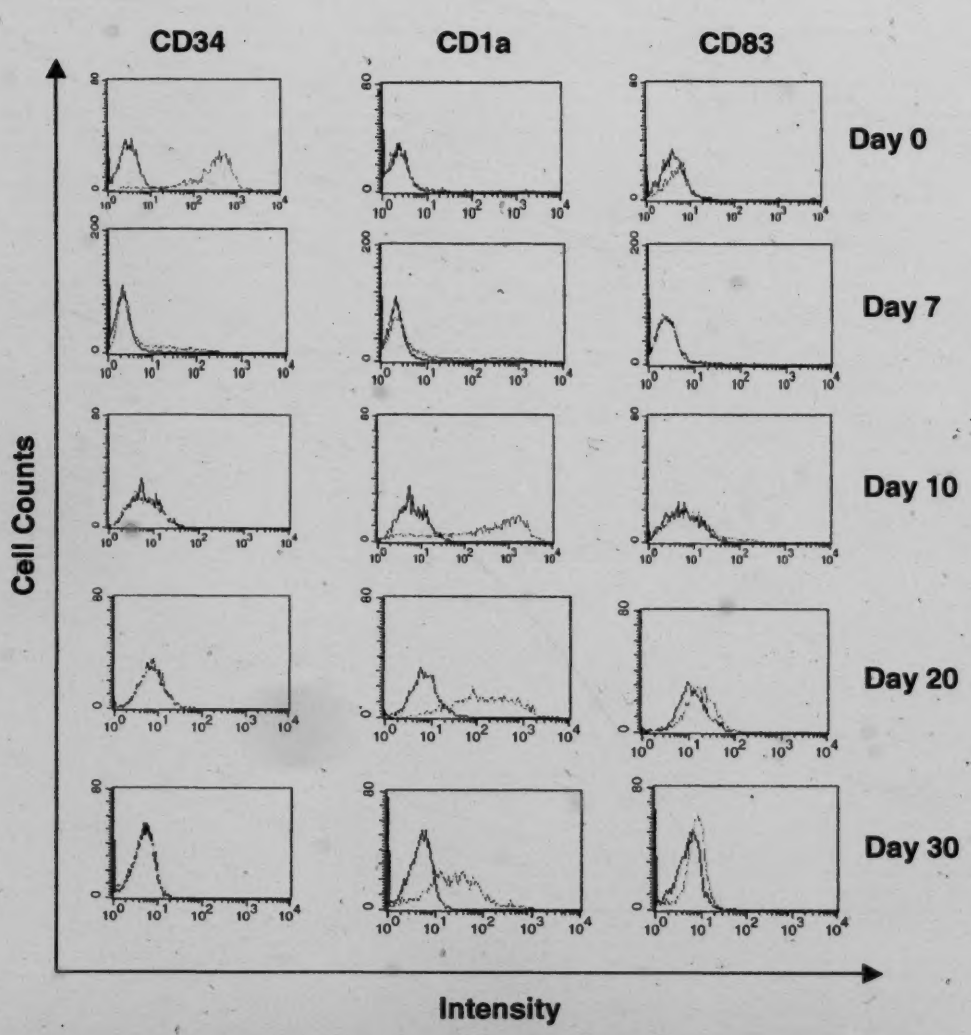

Figure 4-4. Flow cytometry histograms for a representative sample of CD34 progenitor cells isolated from human cord blood and cultured for 30 days. GM-CSF and TNF-α were present in the culture throughout the 30 days while SCF was present for the first 5 days of culture only. The black lines represent the isotype control, the purple lines represent the test condition.

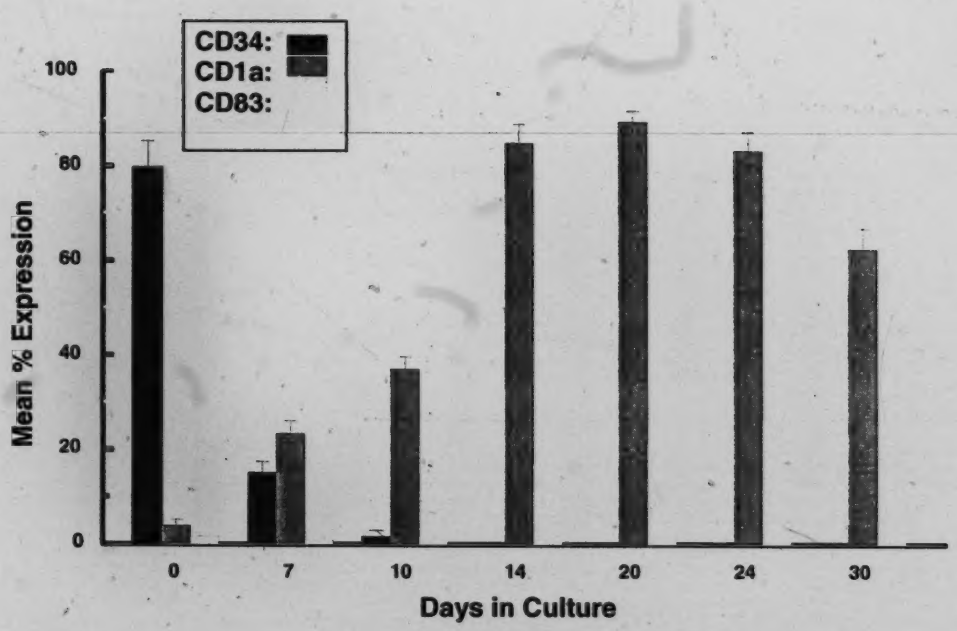

Figure 4-5. The mean percent expression of CD34, CD1a and CD83 on CD34+ progenitor cells isolated from human cord blood and cultured for 30 days in the presence of GM-CSF and TNF-α. SCF was added for the first five days only.

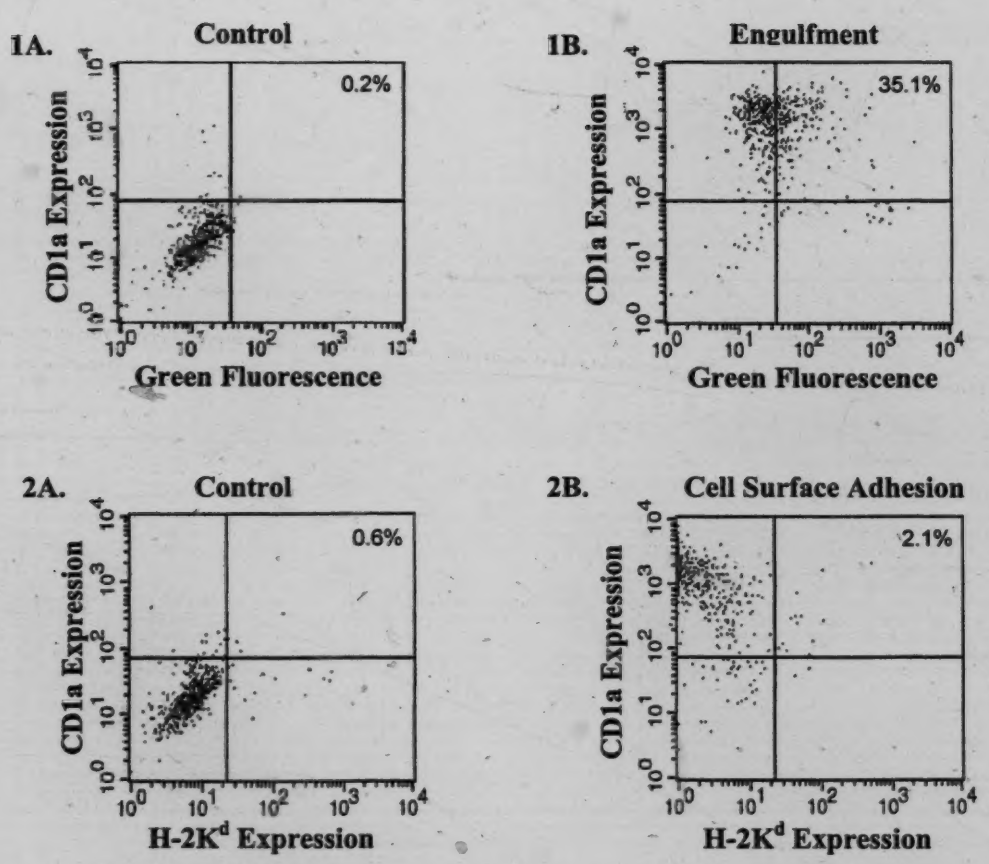

Figure 4-6 The scatter plots showing fragment engulfment versus the adhesion of fragments on the outside cell surface. (1) The co-expression of CD1a and green fluorescence indicates engulfment (upper right hand quadrant). (2) The same group of CD1a+ cells as in (1) simultaneously expressing H-2K^d indicating fragment adhesion to the cell surface (upper right hand quadrant). (A) Isotype control, (B) Fragment and DC co-culture.

CHAPTER FIVE: THE EFFECT OF CPG MOTIF OLIGODEOXYNUCLEOTIDE ON ANTIGEN PRESENTATION BY DENDRITIC CELLS

Introduction

Dendritic Cell Based Immunotherapy

DCs are the most endocytically active cells identified and the most potent APCs with the unique ability to activate naïve T-cells [21]. These properties have made DCs a focal point for cancer immunotherapists in the hopes of developing a cancer vaccine.

The immune system is an attractive target for cancer therapy, as tumors have an inherent ability to evade the immune system. Tumors are highly mutable and therefore can lose or change the expression of surface antigens that are capable of generating an immune response [102]. Tumors do not usually express MHC II, and are capable of down-regulating the cell surface expression of MHC I and co-stimulatory molecules, B7-1 and B7-2 [102,103]. These surface molecules are crucial for antigen presentation to, and the activation of antigen specific T-cells. Tumors have also been shown to secrete various immunosuppressive factors such as, TGF-β, IL-10 and prostaglandin E-2 that have dampening effects on the surrounding immune cells [102]. In fact, DCs isolated from cancer patients have been found to be less immuno-stimulatory than DCs isolated from normal healthy individuals [16,30]. The attempt to override these mechanisms of evasion, thereby making the tumor more noticeable to the immune system, is the principal idea behind cancer immunotherapy.

There are several types of cancer immunotherapy. For instance, the direct administration of IL-2, a cytokine that mediates T-cell expansion, into tumor-bearing mice resulted in tumor regression [4,7,104]. Another strategy involved the transfection of

tumor cells with cytokine genes such as GM-CSF, resulting in a GM-CSF secreting tumor cell. GM-CSF was chosen because of its ability to stimulate anti-tumor immune responses even when transfected in tumor cells [105]. Transfected cells can be injected back into the patient as a vaccine. Parney et al. demonstrated the efficacy of this approach by retrovirally transfecting human Glioblastoma multiforme cells with B7-2 and GM-CSF genes. There was a reduction of tumor growth in an allogeneic human peripheral blood lymphocyte-severe combined immunodeficiency mouse (Hu-PBL-SCID) model [8]. One of the newest, most promising approaches to cancer immunotherapy is DC based immunotherapy.

The principle idea behind DC based immunotherapy is to have DCs recognize and take up tumor specific antigen (TSA) to present to naïve T-cells in order to generate a specific anti-tumor immune response [16]. This most commonly involves *in vitro* isolation of DCs followed by loading of DCs with tumor antigen and finally, re-introduction of the antigen loaded DCs back into the host as a cancer vaccine [16]. Theoretically, the use of true TSA would result in a response directed specifically against tumor cells while leaving normal cells untouched [16].

Using this strategy, bone marrow derived mouse DCs loaded with OVA peptide offered protection in mice from a challenge with a tumor cell line transfected with the OVA gene [53]. Similarly, when human DCs, derived from peripheral blood CD34+ progenitor cells, were loaded with irradiated autologous leukemia cells, both CD4+ and CD8+ T-cells were induced to proliferate and specifically lyse leukemia cells [56]. The success of these, and many other *in vitro* and animal studies have led to several clinical

trials. In particular, DC based immunotherapy clinical trials in melanoma and B-cell lymphoma patients have met with some dramatic successes [3,57].

A melanoma trial performed by Nestle et al. generated DCs from peripheral blood monocytes over a period of 7 days using GM-CSF and IL-4. The DCs were then pulsed with either tumor lysates or a cocktail of immunogenic peptides. KLH was also added as a CD4 helper antigen and as an immunological tracer molecule [57]. The DCs pulsed with tumor lysates or peptides and the KLH constituted the cancer vaccine [57]. Five out of sixteen patients responded to the treatment with two patients experiencing complete regression while three experiencing partial regression. In all cases the vaccine was well tolerated with minimal negative side effects [57].

Hsu et al. performed a B-cell lymphoma clinical trial using dendritic cells isolated directly from the peripheral blood of cancer patients. In each of four B-cell lymphoma patients, idiotype protein was obtained from tumor biopsies. Each patient's DCs were pulsed with the corresponding idiotype for 24 hours, and the DCs were then administered intravenously back into the patient as a vaccine. In other words, each patient received a vaccination with his or her own autologous DCs and idiotype protein. All the patients responded to the vaccine to varying extents. One patient experienced complete tumor regression, one experienced partial tumor regression, a third patient had no sign of the disease as indicated by tumor specific molecular analysis and another remained stable without further need of therapy [3].

<u>CpG Motif Oligodeoxynucleotide</u>

In addition to the DC based therapies described above, the use of immune adjuvants may be useful in DC based cancer immunotherapy to boost the tumor antigen

presenting ability of the DCs to the T-cells. The CpG ODN is one such immune adjuvant. It is a synthetic single stranded ODN with a base sequence modeled on that of bacterial DNA. It contains a relatively high frequency of unmethylated CpG motifs compared to that seen in vertebrate DNA (56). The presence of these CpG motifs is believed to give bacterial DNA its immuno-stimulatory properties [82]. In humans, the CpG motifs are usually present as 5' GTCGTT 3'. As there are a large number of possible CpG ODN sequences, Krieg et al. have systematically screened for the specific CpG ODN sequence that is capable of optimal immune stimulation in humans. This ODN has been called CpG 2006 and has the sequence:

5' TCGTCGTTTTGTCGTTTTGTC GTT 3'

It is preferably phosphorothioate modified to make it more resistant to nuclease degradation [61]. For experimental purposes, a negative control that is commonly used is ODN 2041 with the sequence:

5' CTGGTCTTTCTGGTTTTTTTCTGG 3'

which does not contain any immuno-stimulatory CpG motifs [106].

CpG ODNs are capable of safely inducing a Th1 immune response without the undesirable side effects seen with its counterpart, CFA. CFA is an immune adjuvant that is capable of inducing a potent Th1 immune response. Unfortunately, CFA also has toxic side effects, which limits its clinical use in humans [91]. The identification of an effective and safe Th1 adjuvant is important because a Th1 response activates CTLs that are believed to play a major role in killing modified self cells such as tumor cells and virus-infected cells [103]. The antibody mediated, Th2 response is not as desirable because it involves primarily B-cells that combat foreign pathogens such as bacterial infections [103].

CpG ODNs also have immuno-stimulatory effects on DCs including the maturation and activation of DCs as seen by the up-regulation of cell surface molecules B7-1, B7-2, CD40 and MHC II [16]. In addition to this, in mice, CpG ODNs induce the secretion of immuno-stimulatory cytokines such as IL-12, a Th1 cytokine, and also appear to increase the ability of CTLs to respond to soluble antigen in vivo [85]. Thus, CpG ODNs appear to be an attractive adjuvant for use in humans. It is important to determine whether CpG ODNs are capable of enhancing the antigen presenting ability of DCs.

Purpose of this Investigation

The purpose of this investigation was to determine whether the CD34+ derived DCs were capable of T-cell stimulation, and whether this action was potentiated by the immune adjuvant CpG ODN. To facilitate this study, cells were harvested from CD34+ progenitor cell cultures at day 10. From the studies outlined in Chapter 4, it was determined that the DCs at this stage were immature and capable of antigen capture. A portion of these cells were co-cultured for 24 hours with LT210 cells that had been converted into necrotic cellular fragments through multiple freeze/thaws as described in Chapter 4. LT210 cells are a rapidly proliferating mouse lymphoma cell line that expresses H-2K^d on its cell surface and is thus a xeno-antigen. Following the 24 hour co-culture, the antigen loaded DCs were added to either allogenic or autologous T-cells along with CpG ODN, and cultured for approximately 2 to 3 days to allow proliferation to occur. The end point was determined based on evidence from the cellular proliferation that occurs in lectin- stimulated controls. The amount of proliferation was assessed through the determination of tritiated thymidine uptake by the T-cells.

Materials and Methods

T-Cell Proliferation Assay

Preparation of CD1a+ Cells (Stimulators) and LT210 Necrotic Fragments:

CD34+ progenitor cells were obtained from cord blood as described in Chapter 2. Briefly, MNCs were isolated from cord blood using ficoll-paque density gradient centrifugation and CD34+ progenitor cells were obtained via direct CD34+ immuno-magnetic labeling of MNCs. The CD34+ progenitor cells were then placed into culture with a cytokine cocktail containing 500 U/ml of GM-CSF, 50 U/ml of TNF-α and 50 ng/ml of SCF. These cells were used as stimulators in the T-cell proliferation assay.

LT210 cells were kindly provided by Dr. Kevin Kane (Department of Medical Microbiology and Immunology, University of Alberta). These cells are a mouse lymphoma cell line that expresses H-2K^d on its cell surface. The H-2K molecule is the mouse equivalent of MHC class I in humans.

24 hours prior to the T-cell proliferation assay, 1 X 10^6 non-adherent CD1a+ stimulator cells were removed from 10 day old CD34+ progenitor cell cultures, washed and cultured with necrotic fragments prepared from 1 X 10^6 LT210 cells using repeat freeze/thaws as described in Chapter 4. The final volume of this mixed culture was 30 mls. The stimulators and fragments were co-cultured together for 24 hours in complete RPMI with 200 U/ml of GM-CSF and 50 U/ml of TNF-α at 37°C and 5% CO_2. Complete RPMI contained 10% of either fetal bovine serum or human serum type AB.

Preparation of T-Cells (Responders):

As described in Chapter 2 (Materials and Methods), T-cells that had been isolated via rosetting procedures from cord blood were suspended in 1ml of freezing solution that consisted of 90% fetal bovine or human serum and 10% sterile DMSO. These cells were then cryo-preserved by storing at -70°C overnight before transferring to liquid nitrogen for long term storage. These cells were used as responders in the T-cell proliferation assay.

On the day of the T-cell proliferation assay, responders that were autologous or allogenic to the stimulators were removed from liquid nitrogen and rapidly thawed by constant gentle swirling in a 37°C water bath until almost completely thawed. The responders were then immediately diluted, washed twice and resuspended at a concentration of 1 X 10^6 cells/ml in complete RPMI supplemented with 10% fetal bovine or human serum. All RPMI used to dilute, wash or culture the responders contained 50μM 2-Mercaptoethanol purchased from Sigma Chemicals (Oakville, Ontario).

Set up of T-Cell Proliferation Assay (Stimulators + Responders):

A co-culture of stimulators and responders was prepared at a ratio of 1:10 in a Costar® 96 well flat-bottom tissue culture treated plate from Corning Incorporated (Corning, New York). 100 μl of the irradiated responder cell suspension at 1 X 10^6 cells/ml was added per well of a 96 well plate. Stimulators from 11 day old progenitor cell cultures or the stimulators incubated for 24 hours with necrotic cellular fragments, prepared as described above, were harvested, washed and resuspended at 1 X 10^5 cells/ml in complete RPMI and irradiated with 3000 rads. 100 μl of the appropriate stimulator suspension was added to the responders in the 96 well plate. Experimental conditions

consisted of stimulators cultured alone, responders cultured alone, stimulators co-cultured with responders, stimulators and responders cultured with necrotic LT210 fragments, stimulators, responders and necrotic fragments cultured in the presence of CpG 2006 or ODN 2041, responders cultured with PHA and lastly, responders and stimulators cultured with PHA. These conditions were set up in triplicate.

PHA purchased from Sigma Chemicals (Oakville, Ontario) was diluted to a concentration of 5 µg/50 µl and 50µl of this dilution was added per well. PHA is a lectin with mitogenic activity that polyclonally activates T-cells and is often used as a positive control for T-cell proliferation. Salt purified CpG ODNs (stimulatory and non-stimulatory sequences), were obtained from Operon Technologies (Alameda, California). The stimulatory CpG ODN was referred to as CpG 2006, and its non-stimulatory counterpart was referred to as ODN 2041. The CpG ODNs were diluted to a concentration of 2 µg/ml and 50 µl was added per well. The final volume of each well was 250 µl. Wells that did not receive PHA or ODNs were brought up to volume with complete RPMI.

Stimulators and responders were co-cultured and visually monitored for 1-2 days to observe T-cell proliferation. Once T-cell proliferation was evident in the PHA controls, 50 µCi of tritiated thymidine in 50 µl, were added to each well and after 24 hours of incubation at 37°C and 5% CO2 the cells were harvested and read on a liquid scintillation and luminescence counter. Thymidine was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Access to the TOMTEC cell harvester (Orange, Connecticut), and the Wallac 1450 Microbeta® TRILUX Liquid Scintillation and

Luminescence Counter (Turku, Finland) was kindly provided by Dr. John Samuel (Faculty of Pharmacy, University of Alberta).

Modified T-cell Proliferation Assay: 24Hour Incubation of Stimulators and LT210 Necrotic Fragments in 96 Well Plates:

As mentioned previously in Chapter 4, there was concern that the 30 ml volume used to culture DCs with necrotic cellular fragments was too large to allow optimal antigen uptake and therefore may explain the low percentage of engulfing CD1a+ cells. In response to this concern, an additional experiment was conducted where stimulators were resuspended to a concentration of 1 X 10^5 cells/ml in RPMI supplemented with 10% human type AB serum, and 100 µl were added to each well of a 96 well plate. 1 X 10^6 LT210 cells/ml in RPMI supplemented with human serum were made necrotic through multiple freeze/thaws. 50 µl of this necrotic cell suspension was added per well, to the stimulators in the 96 well plate where required. Both stimulatory CpG (CpG 2006) and its non-stimulatory counterpart (ODN 2041) were diluted to a concentration of 2 µg/ml, and 25 µl were added to each well of the 96 well plate where required. The cells were then incubated for 24 hours at 37°C and 5% CO_2, and irradiated at 3000 rads.

Frozen autologous responders were thawed as described above, washed and resuspended in RPMI supplemented with human serum at a concentration of 1 X 10^6 cells/ml. 100 µl of the responders were added to each well of the 96 well plate. Stimulators and responders were co-cultured and visually monitored for 1-2 days to observe T-cell proliferation. Once T-cell proliferation was evident in the PHA controls, 2500 µCi of tritiated thymidine in 50µl, were added per well and after 24 hours of

incubation at 37°C and 5% CO_2 the cells were harvested and read on a liquid scintillation and luminescence counter.

Results

Statistical significance of differences was calculated using the non-parametric Wilcoxon Test on the counts per minute (cpm) with $p<0.05$ being considered significant. The results of the T-cell proliferation assays are represented as mean cpm and the data is also displayed graphically as stimulation indices (SI). For the autologous T-cell proliferation assays, SIs were calculated by dividing the cpm obtained in each test condition by the cpm of the background condition (autologous DC and T-cell co-culture). It was important to use the DC and T-cell co-culture cpm as the background due to the considerable amount of thymidine incorporation that occurred compared to the negative control of T-cells alone. For the allogenic T-cell proliferation assays, the SIs were calculated by dividing the cpm of each test condition by the cpm of the background condition which, in this case, was taken to be T-cells cultured alone. In the graphs, therefore, the background conditions are given a value of 1.0.

The negative controls for the experiments were T-cells cultured alone. The positive controls were the conditions that involved PHA, a known mitogenic lectin. That is, T-cells alone, cultured with PHA and the co-culture of DCs and T-cells in the presence of PHA. Irradiated DCs cultured alone served to confirm that the irradiation had rendered the responders incapable of proliferation. They therefore did not contribute to the observed thymidine incorporation.

T-Cell Proliferation Assays

Autologous T-Cell Proliferation Assay in Fetal Bovine Serum

The cpm of this study are shown in Table 5-1. A total of 11 cord blood samples were used for this study. The mean cpm and standard error for the background condition was 1,748 cpm ± 710. As mentioned previously, the mean cpm of the background condition was significantly greater (approximately 26 times) than the negative control (66 cpm ± 20, Mean ± SEM; $p<0.01$). Both positive controls were much higher than all other conditions. Interestingly, the SI for the T-cell and PHA condition was 73 ± 39, while the addition of DCs to this condition resulted in a significant decrease in SI (40 ± 22, Mean ± SEM; $p<0.01$).

Compared to the background there was no significant change in SI upon addition of necrotic xenogenic fragments (1.3 ± 0.3, Mean ± SEM; $p=0.7$) (Figure 5-1B), or upon addition of necrotic fragments along with stimulatory CpG 2006 or non-stimulatory ODN 2041 (1.4 ± 0.3; $p=0.09$ and 1.6 ± 0.3; $p=0.2$ respectively, Mean ± SEM). The relative amounts of proliferation in the test conditions compared to background are shown as SIs in Figure 5-1.

The high degree of thymidine incorporation seen in the background co-culture of autologous DCs and T-cells was of concern. A possible contributor to this reaction was thought to be the presence of FBS in the culture medium, which is likely to contain components that are immunologically foreign, resulting in inadvertent stimulation of T-cell proliferation. The magnitude of the background reaction may be masking subtle trends in the data. In an attempt to decrease this background and to ensure that the

presence of FBS was not masking any differences that may be present between conditions, we replaced the FBS in the culture media with human type AB serum and repeated the study.

Autologous T-Cell Proliferation Assay with Human Serum Type AB

Five cord blood samples were studied in this experiment; cpm are shown in Table 5-2. The mean cpm and standard error for the negative control of T-cells alone was 183 cpm ± 36. Unlike the proliferation assays conducted in the presence of FBS, the background counts for autologous DC and T-cells (449 cpm ± 154; Mean ± SEM) were not significantly higher than the negative control of T-cells alone. In other words, when FBS was replaced with human type AB serum, the mean cpm of the autologous background co-culture dropped from approximately 26 times to 2.5 times that of the negative control. Thus, the use of human serum in place of FBS decreased the background cpm significantly and was therefore used in all further tests.

As in earlier studies, the mean cpm of the positive controls were much greater than all other conditions. Again, the addition of DCs to the T-cells in the presence of PHA resulted in a significant decrease SI (98 ± 64 versus 71 cpm ± 56, Mean ± SEM; $p<0.05$).

SIs are used to compare the relative effects of the addition of xenogenic fragments, CpG 2006 and ODN 2041 by standardizing the results according to the autologous background condition (Figure 5-2). There was no statistically significant increase in SI with the addition of necrotic xenogenic fragments alone or fragments along with stimulatory CpG 2006 or non-stimulatory ODN (1.3 ± 0.3, 4.8 ± 2.6 and 6.2 ± 3.4 respectively, Mean ± SEM; $p>0.5$). However, it should be noted that while adding

fragments alone had very little effect, the addition of CpG 2006 or ODN 2041 increased the SI to approximately 5 times that of the background condition. The lack of statistical significance is most likely due to the large variation in results. In the proliferation assays with FBS, the addition of the necrotic fragments and CpG 2006 or ODN 2041 only resulted in SI increases between 1.6 to 1.7 times that of the background condition. Thus, the reduction of background counts obtained with the use of human serum appeared to reveal larger differences in thymidine incorporation between test groups and the background group.

As mentioned in Chapter 4, DCs were cultured with xenogenic necrotic fragments for 24 hours to promote antigen uptake. However, the large 30 ml volume of these cultures was thought to be less than optimal for promoting interactions between DCs and fragments, perhaps resulting in lower antigen uptake and consequently low T-cell stimulation. To examine this, the volume of media used for the culture of DCs and necrotic cellular fragments was significantly reduced from 30mls in a tissue culture flask to 175 µl in a well of a 96 well plate.

The cpm obtained from this experiment are shown in Table 5-3. The SIs are represented in Figure 5-3. Two samples were collected for this experiment, which made it difficult to determine the presence of statistically significant differences. However, the addition of necrotic fragments along with either CpG 2006 or ODN 2041 resulted in an SI increase over background that was greater than when the larger 30 ml volume was used. Therefore, from preliminary results, we conclude that the smaller volume allows CpG 2006 and ODN 2041 to better exert their effects and bring about maximum T-cell proliferation. However, further studies must be done to confirm this.

Although the use of human type AB serum appeared to decrease the autologous background counts and improve the SI of the conditions with CpG 2006 and ODN 2041, the addition of xenogenic fragments did not significantly cause an increase in T-cell proliferation. This was somewhat surprising as DCs are expected to take up and present foreign fragments to T-cells. As a result, we wondered whether the T-cells were not responding to antigen since they were isolated from cord blood and may be functionally immature. To try and determine whether this was the case, an allogenic T-cell proliferation assay was conducted. T-cells should recognize and proliferate in response to the foreign MHC complexes on the surface of the allogenic DC regardless of the ability of the DC to process exogenous antigen into peptide fragments and present them on the cell surface in the context of MHC.

Allogenic T-Cell Proliferation Assay with Human Serum Type AB

The cpm of this experiment are shown in Table 5-4. The SIs are represented graphically in Figure 5-4.

Six cord blood samples were studied in this experiment, and the mean cpm and standard error of the background condition (T-cells cultured alone) was 121 cpm ± 30. Similar to the previous experiments, the positive controls had much higher SIs than any other condition. The SIs of the T-cells and PHA were 268 ± 57. Once again, even though this included an allogenic combination, the addition of DCs significantly reduced the SI (141 ± 28, Mean ± SEM; $p<0.05$).

As expected, the thymidine incorporation in the co-culture of allogenic DCs and T-cells had significantly greater SIs than the background condition (T-cells only) (13 ± 3, Mean ± SEM, $p<0.05$). Therefore, it appears that the T-cells isolated from cord blood are

fully functional due to their ability to respond to the foreign MHC complexes on the surface of the DC. The addition of stimulatory CpG 2006 and non-stimulatory ODN 2041 to the allogenic DC and T-cell co-culture resulted in an SI only 1.2 to 1.4 times greater than the allogenic background co-culture. This increase did not reach statistical significance.

In each of the above detailed proliferation assays, the addition of necrotic fragments along with immuno-stimulatory CpG 2006 or non-immuno-stimulatory ODN 2041 resulted in increases in SIs compared to the negative controls and background conditions. However, it was not clear whether the resultant proliferation was antigen specific or non-specific in nature. To examine this, T-cells were cultured with either CpG 2006 or ODN 2041 in a volume of 175 µl with human serum. The results were a cpm of 4,339 and 3,081 respectively. This results in SIs of approximately 10.6 to 14.9 using T-cells alone as the background. Thus, the presence of the ODNs appears to exert a non-specific effect on T-cells to some degree. However, when DCs were added to the T-cells cultured with ODNs, the cpm increased further by approximately 3.6 times. In conclusion, while CpG 2006 and ODN 2041 exert a considerable non-specific effect on T-cells, they also appear to enhance the T-cell proliferation in response to DCs. More experiments must be conducted to investigate this further.

Discussion

These studies were initially conducted to investigate the ability of DCs to present antigen to T-cells, and to determine whether the addition of the immuno-stimulatory adjuvant, CpG 2006, would enhance the T-cell proliferation stimulated by the DCs. However, in the process of conducting these studies, many additional questions arose.

For example, the use of FBS in the culture medium was thought to distort the results obtained. Most researchers have used FBS or FCS in the culture medium when generating DCs [32;39;98;100], however, Borràs et al. acknowledge that the foreign serum antigens could contribute significant variables that are unaccounted for [96]. It appears to be especially important to use human serum instead of FBS when conducting T-cell proliferation assays [83].

In our study, we found that the mean cpm of the autologous background condition of DCs and T-cells was significantly greater than the negative control in the proliferation assays conducted in the presence of FBS. Since both the DCs and the T-cells were from the same donor sample, the cell populations should not react to one another. One possible explanation was that the presence of FBS in the culture medium was providing foreign antigen that was taken up by the DCs, presented to and recognized by the TCR of the T-cells. To discern whether this was the case, the FBS in all culture media was replaced with human serum type AB. The use of human serum resulted in a considerable decrease in the autologous response so that it was no longer significantly greater than the negative control of T-cells alone. It also enhanced the increases in cpm obtained with CpG 2006 and ODN 2041 over the background condition.

A surprising finding was that the addition of DCs caused a consistent and significant decrease in the response of T-cells to PHA. This suggests that the DCs are having an immunosuppressive effect on the T-cells. Indeed, inhibitory DCs have been reported by other laboratories [25;26]. Chakraborty et al. indicate that inhibitory DCs express lower levels of co-stimulatory molecules and secrete IL-10 while stimulatory DCs express higher levels of co-stimulatory molecules and secrete IL-12. Thus, to

determine whether inhibitory DCs are present, Enyzme Linked Immunosorbant Assay (ELISAs) for IL-10 and IL-12 conducted on the cell supernatants may be helpful.

Another explanation for the decrease in T-cell proliferation after addition of DCs may be that PHA and the DCs induce T-cell proliferation through the same receptor. Thus, due to competition with each other, and because DCs do not have as potent a stimulatory effect on T-cells as PHA, the proliferation appears to decrease.

The lack of a significant response upon addition of the xenogenic fragments was disappointing as these are foreign substances that should be taken up by the DCs to be presented to T-cells. The lack of stimulation suggested several possibilities. Firstly, the DCs may be impaired in the uptake, processing and/or presentation of xenogenic antigen to T-cells. We have shown in Chapter 4 that the xenogenic cellular fragments are taken up by DCs. However we cannot rule out the possibility that subsequent antigen processing or antigen presentation is impaired.

Secondly, the T-cells isolated from cord blood may be too immature to respond appropriately to antigen presentation by DCs. The magnitude of the increase in SI that was calculated for the allogenic co-culture of DCs and T-cells indicated that the T-cells isolated from cord blood are functional and fully capable of responding to the foreign MHC complex on the DC surface.

Lastly, DCs may be presenting antigen but no T-cells of the appropriate specificity are present or appropriate T-cells may be responding but the magnitude of the response may be too small to observe. This could occur if the precursor frequency, that is the number of T-cells capable of responding to this particular antigen, was very low. As a result, the observed T-cell proliferation is less than what would be seen if more T-cells

specific for the xenogenic antigen were present as in a memory T-cell response. We did not investigate the question of precursor frequency. However, the performance of antigen specific CTL assays may be helpful to demonstrate a specific immune response that is of too small a magnitude to be evident in a proliferation assay.

Although the addition of CpG 2006 resulted in considerable increases in SI, the high degree of variation between samples prevented these values from reaching statistical significance when compared to the background condition. An increase in sample number may decrease the degree of variation and result in CpG 2006 achieving statistical significance.

In the allogenic T-cell proliferation assay with human serum, the addition of CpG 2006 to DCs and T-cells resulted in only a marginal increase in SI compared to the background co-culture of T-cells and DCs. The increase observed upon addition of CpG 2006 appeared to have both a non-specific effect on T-cells as well as an antigen specific effect in the small number of tests performed. This is inconsistent with current literature which generally indicates that CpG ODNs are not capable of directly stimulating T-cells. Bauer et al. report that T-cells are not responsive to CpG ODN [83] and reviews by Krieg also report that although the effects of CpG ODN on T-cells is unclear, most evidence points towards the inability of CpG ODN to directly stimulate T-cells [73 91]. This discrepancy must be investigated further in our studies by conducting more experiments on the effect of CpG ODN on T-cells alone.

The stimulatory effect of ODN 2041 was unexpected although not unexplainable. There have been numerous reports that the phosphorothioate backbone of the ODNs can have non-specific stimulatory effects on human DCs [64;106]. Hartmann et al. show that

high concentrations of phosphorothioate oligonucleotides have a CpG-independent background activity [106]. Similarly, Krieg et al. acknowledge that at high concentrations, the phosphorothioate backbone can have intrinsic non-specific immune stimulatory effects especially on human B-cells and DCs [64]. However, this drawback has to be weighed against the benefits of prolonging the half-life of the ODNs by phosphorothioate modification.

As mentioned in Chapter 4, the volume used for the 24 hour incubation between the DCs and xenogenic fragments may have been too large to allow for optimal uptake of antigen. Therefore, an experiment was carried out where incubation took place in a total volume of 175 µl of media in a 96 well plate as opposed to a flask with a volume of 30 mls. The decrease in volume did not result in an increase in response with the addition of fragments alone. However, with the addition of CpG 2006 or ODN 2041, the increase in SI was much greater than the increase seen in the 30 ml condition. Although statistical analysis was difficult to do in this case due to the small sample numbers, it appeared that the smaller volume improved the immuno-stimulatory effect of the ODNs on the DCs. However we cannot rule out the possibility that antigen uptake and processing may be a problem or the low precursor frequency as mentioned previously. More experiments are needed to further elucidate the nature of the interactions.

In conclusion, human serum is favored over FBS as it decreases the magnitude of the autologous reaction and consequently reveals an increased response towards CpG 2006 and ODN 2041. CpG 2006 and ODN 2041 had both non-specific and specific stimulatory effects. Although others have reported that CpG 2006 is immuno-stimulatory and ODN 2041 is non-immuno-stimulatory [106], in our experiments, both ODNs had very

similar effects. This immuno-stimulatory effect resulted in a considerable response compared to the background condition however it did not reach statistical significance. Increasing sample numbers may help alleviate this problem.

The failure of xenogenic necrotic fragments to elicit a proliferation response did not appear to be due to the inability of DCs to take up antigen or the inability of the T-cells to respond to antigen presentation by the DCs. Further studies need to examine details of antigen processing and presentation by the DCs to determine if either or both may be impaired. Other important future studies would be the performance of killing assays to reveal minor degrees of proliferation that are antigen specific in nature.

Conditions								
Sample #	S	R (-)	S+R (B/G	S+R+F	S+R+F 2006	S+R+F 2041	R+PHA (+)	S+R +PHA (+)
1	61	46	2609	2808	3333	3460	45307	7184
2	75	79	981	784	1143	912	45399	22890
3	13	85	759	436	789	557	27944	9373
4	21	24	1955	1899	2672	3544	22988	18701
5	53	75	8511	6342	5089	7403	67488	23849
6	18	21	1091	1195	1002	1055	35879	25279
7	23	25	897	1048	1219	1746	25267	12952
8	11	56	822	1335	1319	1629	33279	22865
9	18	25	1343	1353	1465	1618	39894	22834
10	20	45	221	827	868	968	22906	12045
11	14	23	42	79	56	71	18896	10647
Mean	68	66	1748	1646	1723	2088	35022	17147
STDev	65	65	2356	1719	1433	2072	14114	6761
SEM	20	20	710	518	432	625	4255	2038

Table 5-1. The counts per minute representing the degree of T-cell proliferation in an autologous T-cell proliferation assay using media supplemented with FBS. S=Stimulators (DCs), R=Responders (T-Cells), F=Fragments (LT210 Necrotic Fragments), 2006=CpG 2006 (Immunostimulatory CpG ODN), 2041=ODN 2041 (Non-Immunostimulatory ODN), PHA=Phytohemagglutinin. The negative control is indicated by (-), the positive controls are indicated by (+) and the background condition is indicated by (B/G).

	Conditions							
Sample #	S	R (-)	S+R (B/G)	S+R+F	S+R+F 2006	S+R+F 2041	R+PHA (+)	S+R+PHA (+)
1	87	181	156	283	1821	2726	54934	45674
2	123	316	229	295	151	267	3113	913
3	92	177	490	940	482	488	10885	1403
4	77	128	1023	631	185	979	31735	1246
5	42	114	348	316	3583	3630	26216	18511
Mean	84	183	449	493	1244	1618	25377	13549
STDev	29	80	345	289	1475	1482	20127	19463
SEM	13	36	154	129	660	663	9001	8704

Table 5-2. The counts per minute representing the degree of T-cell proliferation in an autologous T-cell proliferation assay using media supplemented with human serum type AB. S=Stimulators (DCs), R=Responders (T-Cells), F=Fragments (LT210 Necrotic Fragments), 2006=CpG 2006 (Immunostimulatory CpG ODN), 2041=ODN 2041 (Non-Immunostimulatory ODN), PHA=Phytohemagglutinin. The negative control is indicated by (-), the positive controls are indicated by (+) and the background condition is indicated by (B/G).

Conditions								
Sample #	S	R (-)	S+R (B/G)	S+R+F	S+R+F 2006	S+R+F 2041	R+PHA (+)	S+R+PHA (+)
1	225	165	318	344	1526	1320	15223	13776
2	99	348	587	574	15325	12096	23782	16269
Mean	162	257	453	459	8426	6708	19503	15023
STDev	89	129	190	163	9757	7620	6052	1763
SEM	63	92	135	115	6900	5388	4280	1247

Table 5-3. The counts per minute representing the degree of T-cell proliferation in an autologous T-cell proliferation assay using media supplemented with human serum type AB in a volume of 175µl. S=Stimulators (DCs), R=Responders (T-Cells), F=Fragments (LT210 Necrotic Fragments), 2006=CpG 2006 (Immunostimulatory CpG ODN), 2041=ODN 2041 (Non-Immunostimulatory ODN), PHA=Phytohemagglutinin. The negative control is indicated by (-), the positive controls are indicated by (+) and the background condition is indicated by (B/G).

	Conditions						
Sample #	S (-)	R (B/G)	S+R	S+R 2006	S+R 2041	R+PHA (+)	S+R +PHA (+)
1	62	50	382	423	682	25585	11890
2	99	72	2074	1114	1921	19920	8434
3	50	110	607	730	952	15284	4672
4	172	123	1529	1635	1830	23110	12093
5	168	110	1145	2636	2553	35685	19235
6	87	260	4081	7681	8063	42952	45335
Mean	106	121	1636	2370	2667	27089	16943
STDev	52	73	1346	2716	2730	10338	14720
SEM	21	30	549	1109	1115	4220	6009

Table 5-4. The counts per minute representing the degree of T-cell proliferation in an allogenic T-cell proliferation assay using media supplemented with human serum type AB. S=Stimulators (DCs), R=Responders (T-Cells), 2006=CpG 2006 (Immunostimulatory CpG ODN), 2041=ODN 2041 (Non-Immunostimulatory ODN), PHA=Phytohemagglutinin. The negative control is indicated by (-), the positive controls are indicated by (+) and the background condition is indicated by (B/G).

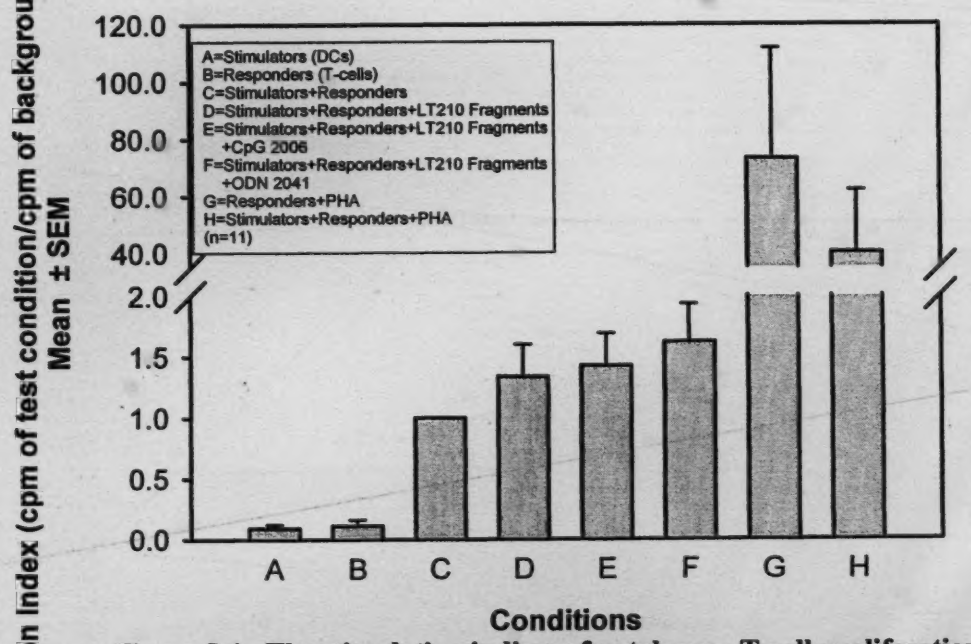

Figure 5-1. The stimulation indices of <u>autologous</u> T-cell proliferation assay conducted in the presence of FBS. The negative control is represented by B, and the two positive controls are G and H. A indicates that the irradiated DCs do not contribute to the observed proliferative response. There is a considerable autologous response in the background condition that results in a significant increase in T-cell proliferation compared to the negative control.

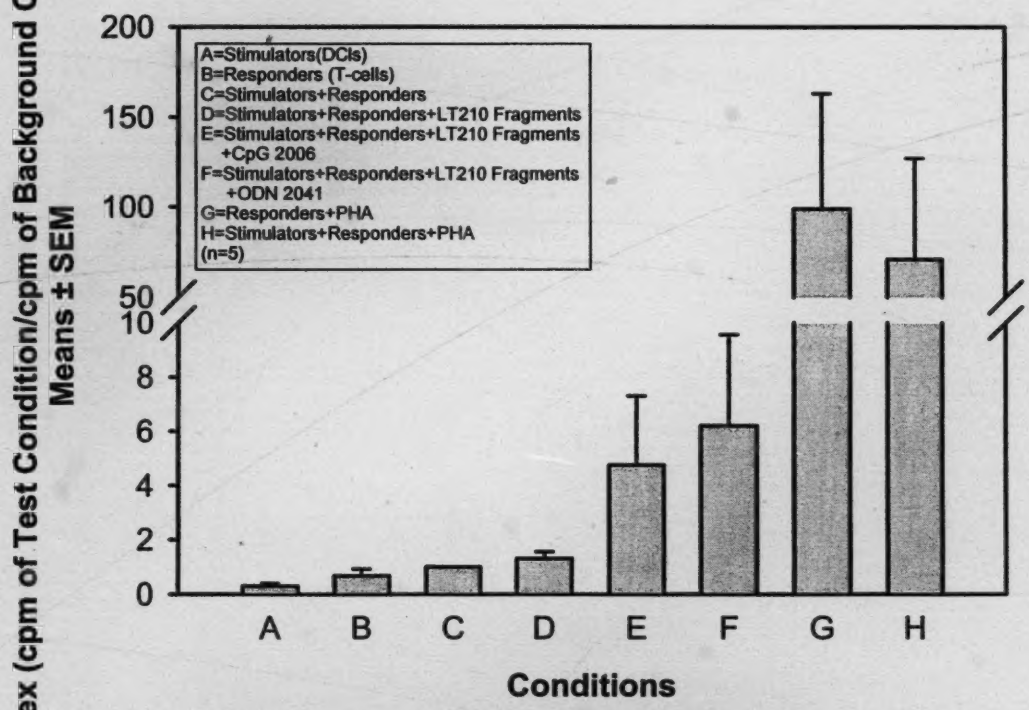

Figure 5-2. The stimulation indices of <u>autologous</u> T-cell proliferation assay conducted in the presence of human serum type AB. The negative control is represented by B, and the two positive controls are G and H. A indicates that the irradiated DCs do not contribute to the observed proliferative response. The autologous response of the background condition is no longer significantly greater than the negative control.

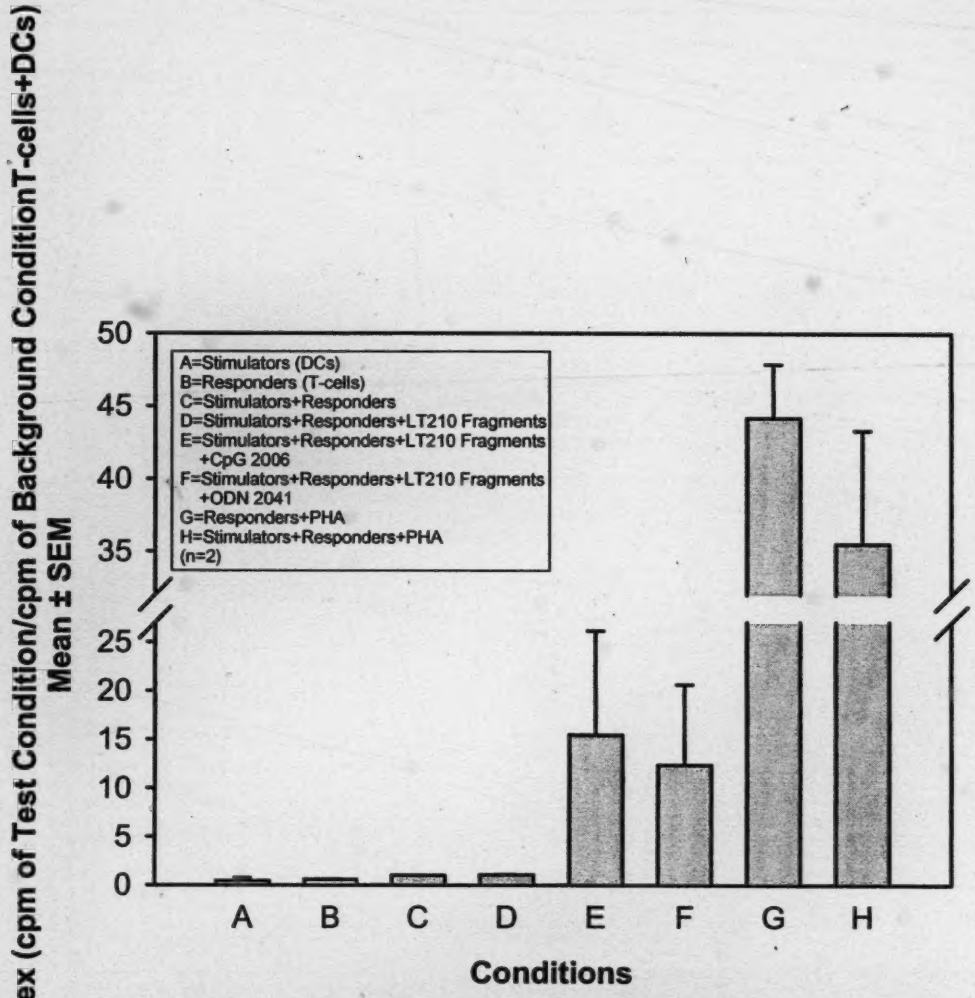

Figure 5-3. The stimulation indices of <u>autologous</u> T-cell proliferation assay conducted in the presence of human serum type AB in a volume of 175µl. The negative control is represented by B, and the two positive controls are G and H. A indicates that the irradiated DCs do not contribute to the observed proliferative response. The autologous response of the background condition is not significantly higher than the negative control, and CpG 2006 (E) and ODN 2041 (F) induce high levels of T-cell proliferation.

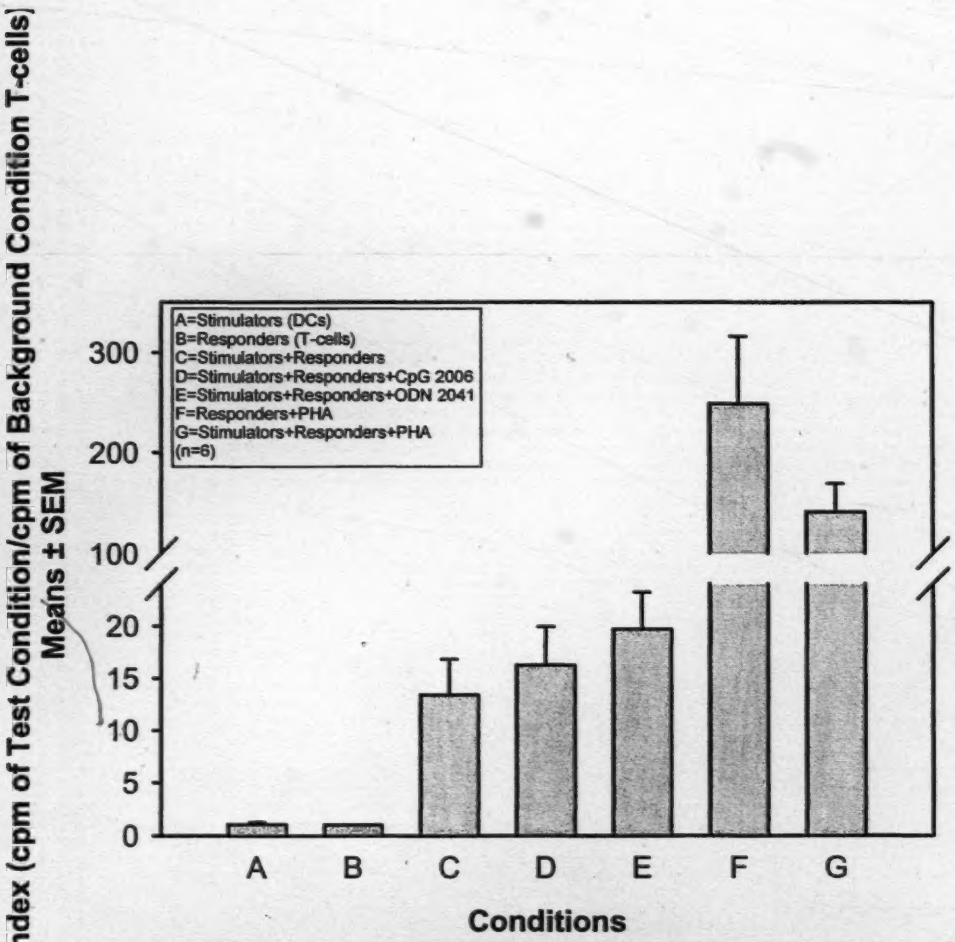

Figure 5-4. The stimulation indices of <u>allogenic</u> T-cell proliferation assay conducted in the presence of human serum type AB. Stimulation indices were calculated by dividing the various conditions by the background condition (A). The negative control is also represented by A, and the two positive controls are F and G. B indicates that the irradiated DCs do not contribute to the observed proliferative response. The allogenic response of DCs+T-cells (C) is significantly greater than the negative control.

CHAPTER 6: GENERAL DISCUSSION

DCs have captured the interest of many researchers due to their potential application in cancer immunotherapy. The endocytic activity and potent antigen presentation ability of DCs has given rise to a rapidly expanding field known as DC based cancer immunotherapy. This primarily involves the pulsing of DCs with tumor antigen so that DCs can take-up, process and present the antigen to lymphocytes in order to generate a tumor specific immune response. The procedure has resulted in several clinical trials that have included prostate cancer, B-cell lymphoma, melanoma, and glioma patients [3,56,107,108]. Attempts to improve the antigen presenting ability of DCs have also been made by using immune adjuvants such as CpG motif ODNs (CpG ODN) which have been studied in great detail by Krieg et al [109].

From the first official characterization of DCs in 1973 by Steinman and Cohn [110], initial research regarding DCs was hampered due to the inability to isolate these cells in large homogenous quantities, and by the absence of a known specific cell surface marker to definitively identify DCs [18,19]. The development of new methodologies, such as immuno-magnetic labeling, and the discovery of new sources of dendritic cells, have helped to circumvent some of these difficulties. Presently, there are three main sources from which DCs can be obtained: peripheral blood, cord blood and bone marrow. Of these sources, peripheral blood is the most convenient, where DCs can be induced to differentiate from peripheral blood monocytes using GM-CSF and IL-4 [17]. However, cord blood is proving to be an equally important and valuable source of DCs.

CD34+ progenitor cells are found at relatively high proportions in cord blood [19] and can be induced to differentiate into DCs using GM-CSF and TNF-α [52]. Dendritic

cells generated this way may be more favorable than DCs generated from peripheral blood monocytes for several reasons. First, the reversion of monocyte derived DCs back into monocytes is always a concern, and indeed, this has been observed to occur following the removal of the appropriate cytokines (GM-CSF, IL-4) [52]. Second, there is evidence that CD34+ progenitor cell derived DCs are slightly more potent at antigen presentation than monocyte derived DCs [94].

In addition, tumor cells have been reported to secrete immunosuppressive factors as a mechanism to avoid immune detection [4]. As a result, dendritic cells isolated from cancer patients have been observed to be less immuno-stimulatory than DCs isolated from normal healthy patients [2,20]. Thus, obtaining DCs indirectly, that is by inducing the *in vitro* differentiation of DCs from monocytes or CD34+ progenitor cells isolated from cancer patients, may be one way of circumventing the immunosuppressive effects of the tumor [96].

Although the methods used to generate DCs in our studies were based on those of Rosenzwajg et al., we felt it was important to optimize this process. To date, a thorough examination of the effects that the quality of the cord blood sample has on MNC and CD34+ progenitor cell yields has not been done. Of the few studies done, Syme et al. showed that storing peripheral blood overnight at 4°C resulted in a decrease in the total number of PBMCs obtained, decreased the expression of MHC II on the DC surface and prevented development of characterisitic DC morphology compared to peripheral blood stored at room temperature [38].

Possible factors that may affect the quality of the cord blood sample can include things such as the method of storage of the collected cord blood sample, the volume of

cord blood obtained from the umbilical cord, if there were complications during delivery and if the cord blood sample was collected immediately after birth and transection of the umbilical cord.

In our study, we used positive immuno-magnetic labeling to isolate CD34+ progenitor cells from cord blood as a relatively pure population. We examined the effects that cord blood volume and the time elapsed between cord blood collection and cord blood processing had on total MNC and CD34+ progenitor cell yields, as well as MNC and CD34+ progenitor cell yields per milliliter of cord blood. We found that in order to generate optimal viable CD34+ progenitor cell yields, cord blood samples with large sample volumes were best even though the larger volumes appeared to contain a smaller fetal blood to maternal blood ratio. Also, viable CD34+ progenitor cells could be obtained from cord blood samples as long as 48 hours after collection. Conversely, MNC yields were best obtained within 0 to 11 hours after collection, perhaps suggesting that CD34+ progenitor cells are more resilient than the MNC population. The ability to obtain a relatively pure population of CD34+ progenitor cells from cord blood meant that our laboratory was supplied with a reliable source of human DCs.

In addition to the effects of factors occurring in vitro, it would be interesting to determine the effect of factors in the clinical setting such as complications during delivery, and the length of time elapsed between umbilical cord transection and collection of the cord blood sample if cord blood is collected immediately after delivery and umbilical cord transection . Controlling these factors, such that an optimal sample is obtained, coupled with the use of SCF and FLT3L, would allow us to potentially maximize the amount of MNCs and CD34+ progenitor cells obtained from each sample.

We used umbilical cord blood because it was a convenient source of CD34+ progenitor cells for *in vitro* studies in the laboratory. However, umbilical cord blood is obviously not available in adult cancer patients and the use of unrelated cord blood is not feasible because of the issues of allograft rejection. To obtain autologous CD34+ progenitor cells from cancer patients, mobilization can be done, whereby G-CSF is injected intravenously into the patient causing the movement of CD34+ progenitor cells from the bone marrow into the peripheral blood. This results in a substantial increase in the percentage of CD34+ progenitor cells found in the peripheral blood which can then be collected for isolation of the CD34+ progenitor cells.

As mentioned previously, the identification of DCs was initially difficult due to the absence of a known cell surface marker unique to DCs. The recently discovered DEC-205 and DC-SIGN molecules are two possible candidates for DC specific markers, however, a combination of morphological, phenotypic and functional characteristics is still favored for characterizing DCs [17,35 27-29]. Morphologically, DCs are quite unique. They exhibit numerous projections and protrusions on their cell surface reminiscent of dendrites found on neurons of the nervous system. Previously, the DC phenotype of surface markers was not considered unique and only by considering a constellation of cell surface markers could the presence of DCs be determined. The recent discovery of DEC-205 and DC-SIGN may help to resolve this hurdle, but the DC surface markers identified have included CD1a, CD4, CD40, CD54 (ICAM-1), CD58 (LFA-3), CD80, CD86 and CD83 [17,35]. DCs also express high levels of MHC I and MHC II, which are further up-regulated upon DC activation [16,30]. Mature activated DCs usually express CD83 [16,30]. These cell surface markers can all be found on other cells of the immune system,

although in varying amounts. Even CD1a, which is considered to be one of the principle identifying markers of DCs can be found on some macrophages [52]. Functional characterisitics can also be used to identify DCs. One prime example is their efficient endocytic activity, a trait that makes DCs an attractive target for cancer immunotherapy. Additionally, functional identification can be made on DC ability to present antigen to T-cells.

In our study, we used cellular morphology, cell surface phenotype and functional studies to positively identify the cells generated from CD34+ progenitor cells using GM-CSF, TNF-α and SCF as DCs. Morphologically, the cells began to develop processes after approximately 5 days in culture with the cytokines. These cytoplasmic protrusions were more evident after approximately 10 days in culture. By day 7 the formation of cell colonies was evident increasing in size by day 10 and becoming adherent by day 14. In terms of phenotype, the cells lost most of their CD34 expression by day 10; CD1a expression was prominent by day 10 and peaked at day 20. CD83 expression was up-regulated by day 14 and also peaked at day 20. In addition to this, the CD1a+ cells also expressed high levels of CD40 and MHC II. However, although the cell markers continued to increase up to day 20, total cell numbers began to drop dramatically after day 14. For this reason, coupled with the fact that CD83 expression began to appear at day 14 (indicating maturation), we chose to harvest the cells at day 10 since we needed to use immature DCs for our studies.

It is important to note that DCs have different functional abilities at different maturational stages. For example, DCs are very endocytically active in their immature stage. However, as they mature, the DC begins to lose its endocytic ability and becomes

specialized in antigen presentation [19]. Thus it was important to identify immature DCs for the functional component of DC identification. Most studies use the ability of DCs to take up antigen as an indicator that the cells are in an immature state however few studies have examined the maturational stages using cell surface phenotype.

In our experiment, by observing the down-regulation of CD34 expression and the onset of CD1a and CD83 expression we were able to determine when CD34+ progenitor cells made the transition to immature DCs, and when the immature DCs transitioned to mature DCs. We confirmed the presence of immature DCs by assessing the ability of these cells to take up antigen.

Based on cell surface phenotype we determined that optimal numbers of immature DCs were present between day 10 and day 14 of culture. The capacity of cells at this stage to take up necrotic xenogenic cellular fragments was assessed and indeed, antigen uptake was present. In summary, by day 10, the cells we derived from CD34+ progenitor cells exhibited characterisitically long cellular projections, expressed CD1a, CD83, MHC II and CD40, and were capable of antigen uptake. They therefore fit the profile of a DC. Our next step was to determine whether the use of the CpG motif ODN immune adjuvant could increase the antigen presenting capabilities of DCs.

The CpG ODN is a single stranded synthetic ODN that has a base sequence derived from bacterial DNA. It contains a high frequency of unmethylated cytosine/guanine (CpG) motifs [61]. Similar to bacterial DNA, certain CpG ODNs have been found to have a wide variety of immuno-stimulatory effects [28;72;73;83;87;91;106]; of interest to our discussion are the immuno-stimulatory effects on DCs. The CpG ODN can induce the maturation and activation of DCs as seen by the up-regulation of cell

surface molecules B7-1, B7-2, CD40 and MHC II [82,83]. Since these markers are all involved in the presentation of antigen and activation of T-cells, it may be possible that the CpG ODN is capable of increasing the antigen presenting ability of DCs.

Due to the large number of possible CpG ODN sequences, Krieg et al. systematically screened for the most immuno-stimulatory CpG ODNs in humans by assessing the ability of various CpG ODNs to activate human B-cells [72]. The most immuno-stimulatory CpG ODN was found to be:

TCGTCGTTTTGTCGTTTTGTC GTT

This sequence is known as CpG 2006, and is the CpG ODN that is used in our study. The non immuno-stimulatory counterpart of CpG 2006, ODN 2041, which does not contain any CpG motifs was used as a control.

From our T-cell proliferation experiments, it does appear that CpG 2006 is capable of increasing T-cell proliferation. However, this increase failed to reach statistical significance, probably due to small sample numbers and/or the large amount of sample-to-sample variability. It cannot be concluded that CpG 2006 causes an antigen-specific enhancement of an immune response due to the presence of a considerable non-specific effect seen when T-cells alone were cultured in the presence of CpG 2006. Although Klinman et al. showed that CpG ODNs are capable of directly stimulating T-cells as measured by the secretion of IL-6, IL-12 and IFN-γ [75], Krieg has reported that most studies have found that there is no direct effect of CpG ODNs on T-cells alone [91 73]. Other studies have suggested that T-cells are not directly activated by CpG ODN but instead act as a co-stimulus for T-cells stimulated via the TCR [79;80]. Bendigs et al. reported that highly purified T-cells stimulated through the TCR exhibited synergistic

effects towards CpG ODN [79]. Thus, there may be some degree of non-specific stimulation serving to enhance the antigen-specific T-cell response [79]. The high degree of T-cell proliferation observed in response to CpG 2006 in our study may also be due to impurities in the population of T-cells. Isolating T-cells using sheep red blood cells, as opposed to more specific methods such as immuno-magnetic labeling may give a less pure population of T-cells. Thus, CpG ODN may be exerting effects on other cells present causing them to proliferate or, in turn be causing the release of cytokines from these cells which stimulate T-cells [73]. Indeed in Klinman's study, they used partially purified T-cells only [75].

The increased magnitude of the proliferative response seen with CpG 2006 was also observed with the non-stimulatory ODN 2041. Thus, it appears that T-cells proliferate non-specifically in the presence of both the ODNs. This may be explained by observations made by other researchers who showed that if used at higher concentrations, the phosphorothioate backbone of the CpG ODN can cause non-specific immuno-stimulation especially of human B-cells and DCs [75;106]. However, in our studies, the concentration of CpG 2006 and ODN 2041 was 2 μg/ml. This concentration was based on a study by Hartmann et al. who did not observe stimulatory effects with the non-CpG control oligonucleotide [106]. Thus, it does not appear that the concentration of ODN that we use in our studies was the explanation for the T-cell proliferation.

Other possible factors that could increase T-cell proliferation may be presence of endotoxin in the media or on the glassware. However, using a limulus amebocyte lysate (LAL) assay, levels of endotoxin in both ODN preparations were negligible (data not shown).

We ruled out the possibilities that the lack of a response upon addition of xenogenic necrotic fragments was not due to the inability of DCs to take up antigen, or to the inability of T-cells isolated from cord blood to respond to antigen. However, we did not directly examine whether antigen was being processed and presented appropriately. Another explanation of the lack of response towards the xenogenic fragments may be a low precursor frequency since we are using naïve T-cells. That is, although the DCs and T-cells may be functioning properly, the number of T-cells capable of responding to this particular antigen may be so low that they are not represented in the proliferation assay or in such minor numbers that the proliferation is below the sensitivity of our thymidine uptake assay.

In conclusion, we have refined and optimized the conditions so that we are confident through morphological, phenotypic and functional analysis that CD1a+ DCs are being generated from human umbilical cord blood. In the future, the question of whether low precursor frequency is playing a role in the lack of response observed upon addition of xenogenic fragments to autologous DC and T-cell co-cultures should be examined. If the response is simply of too low a magnitude to observe in the proliferation assay, killing assays may be helpful. In this case, following the co-culture of DCs and T-cells in the presence of antigen, the cells are then incubated with target cells expressing the same antigen to observe killing. If antigen specific CTLs were generated the targets will be killed. In addition, more proliferation assays conducted with human serum in 96 well plates must be conducted to decrease the variability which may be responsible for the lack of statistical significance upon addition of CpG 2006 and ODN 2041.

The huge variability in proliferative responses between samples may also be due to the generation of two different types of populations of DCs. Chakraborty et al. has reported the development of two different populations of DCs under identical culture conditions [25,26]. One population, identified as inhibitory DCs, secreted a larger amount of IL-10 and expressed fewer co-stimulatory molecules on the cell surface. The other population was considered stimulatory DCs and secreted a larger amount of IL-12 and expressed a larger percentage of co-stimulatory molecules on the cell surface [25,26]. By collecting cell supernatants and doing IL-10 and IL-12 ELISAs, it may be possible to determine whether the huge variability between samples is due to the generation of inhibitory DCs in some samples. Certainly, their presence is suggested by the fact that the PHA stimulation was consistently suppressed with the addition of DCs in our experiments. Lastly, the non-specific effect of CpG 2006 and ODN 2041 on T-cells must be further confirmed by performing more studies with T-cells cultured alone with the ODNs.

We have established a reliable and reproducible method of generating functional CD1a+ DCs from CD34+ progenitor cells. This methodology is directly applicable to DC-based cancer immunotherapy where the CD34+ progenitor cells of cancer patients could be isolated from their own peripheral blood following a mobilization procedure with G-CSF. Alternatively, DCs generated from CD34+ progenitor cells isolated from cord blood provides a source of DCs that can be used for future *in vitro* adjuvant studies.

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